MUSCLE-TENDON-INNERVATION UNIT: DEGENERATION AND AGING – PATHOPHYSIOLOGICAL AND REGENERATION MECHANISMS

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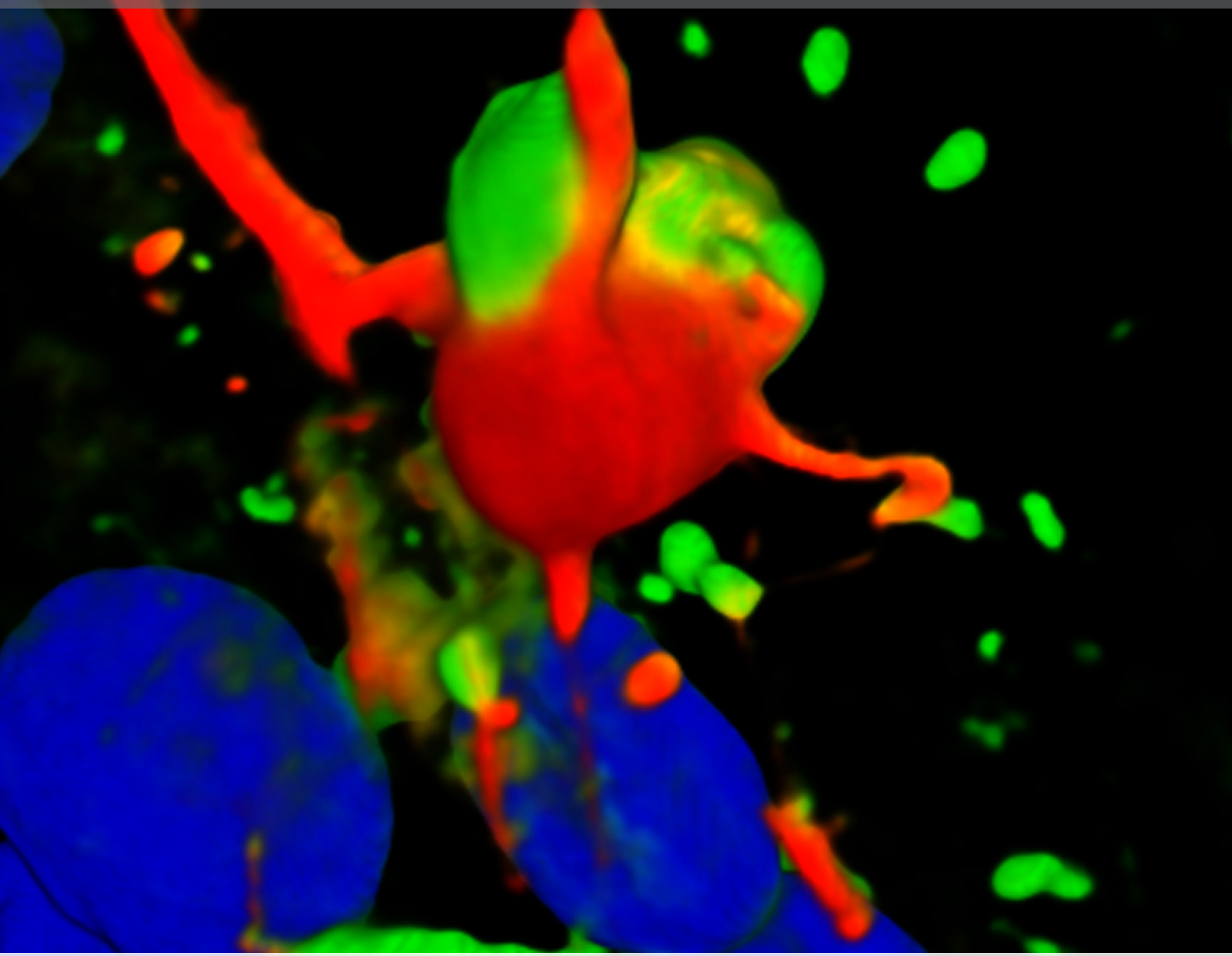

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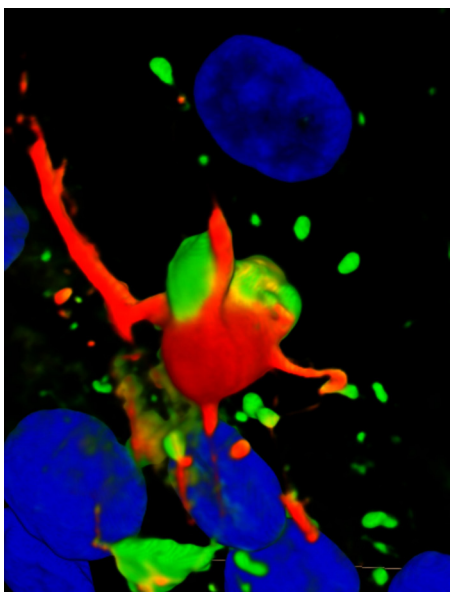
MUSCLE-TENDON-INNERVATION UNIT: DEGENERATION AND AGING – PATHOPHYSIOLOGICAL AND REGENERATION MECHANISMS

Topic Editors:
Luciano Merlini, University of Bologna, Italy
Cesare Faldini, University of Bologna, Italy
Paolo Bonaldo, University of Padova, Italy



Immunofluorescence analysis of cultured tendon fibroblasts from a patient affected by Ullrich congenital muscular dystrophy, labeled with anti-collagen VI (green) and anti-collagen I (red) antibodies. The picture shows an anomalous protein aggregate in the extracellular matrix. Cell nuclei are stained with DAPI (blue).
Cover image by Patrizia Sabatelli, IGM-CNR, c/o IOR, Bologna.

Aging is characterized by progressive deterioration of walking ability. This function loss has multiple causes including central and peripheral nerve dysfunction, loss of muscle mass and strength, as well as joints and bone alterations. Muscle-tendon unit and its innervation has a pivotal role in motor function performance that can be disrupted by overuse degeneration and aging.

Research has shown that overuse degeneration and aging also share some pathophysiological mechanisms including mitochondrial dysfunction, increased apoptosis, abnormal modulation of autophagy, decline in satellite cells, increased generation of reactive oxygen species, and modification of signalling and stress response pathways.

This Research Topic is intended to bring together basic researchers and clinicians working in the area of neuroscience, aging, sarcopenia and orthopaedics in human and in animal models. The aim of this cross-fertilization is to accelerate our understanding of the mechanisms involved in aging and degeneration of the muscle-tendon unit and its innervation and to explore the therapeutic potential of pharmacological and physical therapy interventions.

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EDITORIAL
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Editorial: Muscle-Tendon-Innervation Unit: Degeneration and Aging—Pathophysiological and Regeneration Mechanisms

Luciano Merlini[1]*, *Cesare Faldini*[2]* and *Paolo Bonaldo*[3]*

[1] Department of Biomedical and Neuromotor Sciences, University of Bologna, Bologna, Italy, [2] Department of Orthopedics, Istituto Ortopedico Rizzoli, University of Bologna, Bologna, Italy, [3] Department of Molecular Medicine, University of Padova, Padova, Italy

Keywords: muscle, skeletal, tendons, nerve fibers, aging, degenerative diseases

Editorial on the Research Topic

Muscle-Tendon-Innervation Unit: Degeneration and Aging—Pathophysiological and Regeneration Mechanisms

This research topic brings together basic researchers and clinicians working in the area of neuroscience, aging, sarcopenia, and orthopedics in human and in animal models with the aim to accelerate our understanding of the mechanisms involved in aging and degeneration of the muscle-tendon unit and its innervation and to explore the therapeutic potential of different interventions.

Tendon injuries are the most common cause of chronic pain and temporary/permanent disability in both the world of sport and at work. Tendinopathy, once mostly associated with inflammation and called tendonitis, is now considered the result from an imbalance between protective/regenerative modifications and acute or chronic tendon overload. The treatment modalities of tendinopathy are very different and even conflicting and have in common a lack of scientific evidence. It is therefore important to investigate the basic mechanisms that have translational purposes.

Chen et al. observed in the skeletal muscle of rabbits fed a 2% cholesterol-enriched diet for 12 weeks the presence of abnormally enlarged endolysosomes containing accumulations of free cholesterol and multiple Alzheimer's disease (AD) marker proteins subject to misfolding and aggregation including Aβ, phosphorylated tau, and ubiquitin. These results suggest that elevated levels of plasma cholesterol can alter endolysosome structure and function promoting the development of AD-like pathological features in skeletal muscle, which might contribute to the development of skeletal muscle dysfunction in AD patients (Chen et al.).

Schulz-Schaeffer is proposing that camptocormia in Parkinson's disease (PD), a situation characterized by an anterior flexion of the spine, is related to proprioceptive dysregulation. Camptocormia occurs in several conditions including myopathies, myositis, dystonia, and is particularly frequent in PD. Besides central proprioceptive dysregulation of muscle tone like in PD-associated camptocormia, similar myopathological changes can be observed whether the alterations of the proprioceptive polysynaptic reflex arch occur at the level of the Golgi tendon organ, the dorsal root ganglia, or nerves by disk herniation or aging spondylotic changes (Schulz-Schaeffer).

Vasta et al. reviewed the biological processes involved in tendon healing including the synthesis of collagen and focusing in particular on the role of vascular and neuronal molecular pathways. Vascular endothelial growth factor (VEGF) has shown a key angiofibroblastic role in tendon healing

Edited and reviewed by:
Rodrigo Orlando Kuljiš, University of Miami School of Medicine, USA

***Correspondence:**
Luciano Merlini
luciano.merlini@unibo.it
Cesare Faldini
cesare.faldini@unibo.it
Paolo Bonaldo
bonaldo@bio.unipd.it

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both in animal models and *in vivo* in patients. Recent studies have enlightened the positive role of neurotransmitters like substance P and nitric oxide in the treatment of tendinopathy, supporting the hypothesis of a nerve-mediated dysregulation of tendon metabolism (Vasta et al.).

Yin et al. report that semaphorin 3A is a potent inhibitor of axonal outgrowth and vascular proliferation and has a negative regulation of the matrix metalloproteinases, which are involved in degradation of extracellular matrix proteins of disk. This suggests that semaphorin 3A may act as a potential target for low back pain (Yin et al.).

Rosso et al. reviewed the role of mechanical stimulation by means of extracorporeal shock wave (ESW) and pulsed electromagnetic fields (PEMF) for the treatment of tendinopathy and tendon regeneration. While preclinical and *in vitro* studies suggested effectiveness, the authors pointed out that there is still a lack of strong evidence of the efficacy of these modalities in the clinical settings, suggesting the need of further *in vivo* human studies to confirm the clinical efficacy of mechanical stimulation for the treatment of tendinopathies (Rosso et al.).

Parchi et al. reviewed the various applications of nanoparticles for tendon healing and regeneration: labeling tendon stem cells, acting as carrier for gene therapy and drug delivery, allowing the construction of a new generation of bioactive scaffolds, and modulating the cellular and extracellular matrix response. In their paper, the authors highlighted and summarized the most recent advances and results (Parchi et al.).

Frizziero et al. reviewed the most recent clinical and experimental studies regarding the consequences of detraining in tendon mechanobiology. Overall, the *in vitro* and *in vivo* models showed, after detraining, noticeable alterations of tissue structural organization and of mechanical properties. Although clinical studies showed more variable results, the authors suggest that after a period of sudden detraining physical activity including rehabilitation should be restarted with caution (Frizziero et al.).

Giusti and Pepe described the organization of elastic fibers in tendon and particularly of fibrillin 1 and fibrillin 2 in some animal models and in humans. They review the clinical manifestations of monogenic disorders due to mutations in the genes of fibrillin 1 (Marfan syndrome) and fibrillin 2 (Congenital contractural arachnodactyly), which may cause a combination of joint hypermobility and contractures (Giusti and Pepe).

Popov et al. studied the aged related phenotype of tendon stem/progenitor cells (TSPCs) and its effect on tendon maintenance and healing. Their results showed a significant down regulation of the ephrin receptors EphA4, EphB2, and EphB4 and ligand EFNB1 in aged TCPC. These novel data suggest that decreased expression of ephrin receptors during tendon aging and degeneration limits the establishment of appropriate cell–cell interactions between TSPC and significantly diminishes their proliferation, motility and actin turnover (Popov et al.).

Raz et al. investigated age-associated changes in shoulder muscle pathology combining radiological and histological procedures. They found that patterns of age-associated muscle degeneration were similar between individuals with and without muscle tears. Furthermore, they found that torn rotator cuff muscles display tissue hallmarks of muscle aging, including fatty infiltration, increase in extra-cellular matrix, and loss of slow oxidative myofibers. In contrast, the teres minor exhibited healthy muscle features suggesting that teres minor could represent an aging-resilient muscle (Raz et al.).

Salini et al. compared the efficacy of platelet rich plasma (PRP) therapy in young and elderly subjects suffering for Achilles tendinopathy. They found that PRP is less effective in aged people, suggesting that this can be ascribed to several biochemical and biomechanical differences documented in tendons of young and elderly subjects with the latter showing reduced number and functionality of tenocytes and tenoblasts, which become more evident in the long-term tissue healing (Salini et al.).

Sardone et al. studied a peroneal tendon biopsy and tenocyte culture of a patient with Ullrich congenital muscular dystrophy (UCMD) due to compound heterozygous mutations in the *COL6A2* gene. They found irregular profile and reduced mean diameter of tendon fibrils, and abnormal accumulation of collagen VI and altered distribution of collagen I and fibronectin. In tenocyte culture, collagen VI web formation and cell surface association were severely impaired and metalloproteinase MMP-2 was increased in the conditioned medium. Altogether, these new data indicate that collagen VI deficiency may influence the organization of UCMD tendon matrix, resulting in dysfunctional fibrillogenesis (Sardone et al.).

Finally, we realize that this collection of articles and reviews cannot exhaust all lines of research related to the biology and the pathology of the muscle-tendon complex and its innervation. These articles, however, outline the main pathogenetic mechanisms and thus the possible rehabilitation therapeutic interventions to counteract the aging process of the nerve-muscle-tendon unit. We hope that the items of this collection will be a stimulus for future progress toward a niche of research with great expectations for aging well.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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ORIGINAL RESEARCH
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Role of Endolysosomes in Skeletal Muscle Pathology Observed in a Cholesterol-Fed Rabbit Model of Alzheimer's Disease

Xuesong Chen, John F. Wagener, Othman Ghribi and Jonathan D. Geiger*

Department of Basic Biomedical Sciences, School of Medicine and Health Sciences, University of North Dakota, Grand Forks, ND, USA

Deficits in skeletal muscles contribute not only to the functional decline in people living with Alzheimer's disease (AD), but also to AD pathogenesis. We have shown that endolysosome dysfunction plays an important role in the development of AD pathological features in a cholesterol-fed rabbit model of AD. Interestingly we observed in skeletal muscle from the rabbit AD model increased deposition of Aβ, phosphorylated tau, and ubiquitin. Here, we tested the hypothesis that endolysosome dysfunction commonly occurs in skeletal muscle and brain in this rabbit model of AD. In skeletal muscle of rabbits fed a 2% cholesterol-enriched diet for 12 weeks we observed the presence of abnormally enlarged endolysosomes, in which were increased accumulations of free cholesterol and multiple AD marker proteins subject to misfolding and aggregation including Aβ, phosphorylated tau, and ubiquitin. Moreover, in skeletal muscle of rabbits fed the cholesterol-enriched diet we observed decreased specific activities of three different lysosome enzymes. Our results suggest that elevated levels of plasma cholesterol can disturb endolysosome structure and function as well as promote the development of AD-like pathological features in skeletal muscle and that these organellar changes might contribute to the development of skeletal muscle deficits in AD.

Keywords: Alzheimer's disease, muscle, LDL, endolysosome, amyloid beta, phosphorylated tau, ubiquitin

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Paolo Bonaldo,
University of Padova, Italy

Reviewed by:
Ralf J. Braun,
Universität Bayreuth, Germany
Barry Boland,
University College Cork, Ireland

****Correspondence:***
Xuesong Chen
xuesong.chen@med.und.edu

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INTRODUCTION

Alzheimer's disease (AD) is the most common neurodegenerative disorder of old age that results in massive health care costs in the United States (Hebert et al., 2013). AD is characterized clinically by a progressive cognitive impairment and pathologically by neurodegeneration and the presence of amyloid plaques composed of amyloid beta (Aβ) protein and neurofibrillary tangles composed of phosphorylated tau (Holtzman et al., 2011; Goate and Hardy, 2012). Besides neurodegeneration and cognitive impairment, AD patients often exhibit loss of muscle mass and reduced muscle strength, and such deficits in skeletal muscle may be early signs of AD and as such these deficits may help predict the onset and progression of clinical AD (Buchman et al., 2007; Boyle et al., 2009; Burns et al., 2010). Furthermore, skeletal muscle dysfunction can lead to progressive functional problems in AD patients and some have proposed that these changes may be not only concordant to those

occurring in brain, but also that the skeletal muscle changes may serve as a potential biomarker for the onset and progression of AD (4–6). Although it is not known how such skeletal muscle deficits are developed in AD, a testable hypothesis is that common pathogenic processes occur in brain as well as skeletal muscle and that these changes may help explain observations of elevated levels of Aβ in skeletal muscle of AD patients (Kuo et al., 2000).

Altered cholesterol homeostasis in general and elevated plasma LDL cholesterol in specific continues to represent robust risk factors of sporadic AD (Solomon et al., 2009; Chen et al., 2010; Lesser et al., 2011; Hui et al., 2012; Reed et al., 2014). Others and we have shown that rabbits fed a cholesterol-enriched diet exhibit pathological hallmarks of AD including increased levels of Aβ, phosphorylated tau, and synaptic disruption (Sparks et al., 1994; Ghribi et al., 2006; Chen et al., 2010). Mechanistically, we demonstrated in such rabbits that elevated levels of plasma cholesterol disrupted blood–brain barrier integrity (Ghribi, 2006; Chen et al., 2008a), increased brain levels and the neuronal endolysosome accumulation of apoB (the exclusive apolipoprotein of LDL cholesterol that is normally found only in the periphery), disturbed the structure and function of neuronal endolysosomes, and led to the appearance of pathological features of AD including disrupted synaptic integrity, brain deposition of Aβ, and tau pathology (Chen et al., 2010). Thus, LDL cholesterol coming from the systemic circulation might be altering neuronal endolysosome function and contributing to the pathogenesis of AD. Furthermore, using primary cultured neurons, we demonstrated that LDL cholesterol treatment increased endolysosome accumulation of cholesterol, enlarged the sizes and numbers of endolysosomes, and elevated endolysosome pH (Hui et al., 2012). Moreover, we demonstrated that such alterations in the structure and function of endolysosomes were directly involved in Aβ deposition, tau pathology, and disrupted synaptic integrity (Hui et al., 2012). Thus, our findings suggest strongly that elevated levels of LDL cholesterol contribute to pathogenesis of AD by disturbing the structure and function of neuronal endolysosomes.

Cholesterol for neurons is supplied by astrocyte-derived lipoproteins. Similarly, cholesterol for skeletal muscle is supplied by plasma lipoproteins (Spady and Dietschy, 1983). Extracellular cholesterol, mainly in the form of LDL particles, is delivered into muscle cells via receptor-mediated endocytosis and transported to endolysosomes (Brown and Goldstein, 1986). Importantly, we have shown that the same cholesterol-fed rabbits that develop AD-like pathology in brain (Chen et al., 2010) exhibit increased deposition of Aβ and phosphorylated tau in skeletal muscle fibers (Chen et al., 2008b). Because of the link between LDL cholesterol and endolysosomes and because altered endolysosome dysfunction plays an important and early role in the pathogenesis of AD (Nixon, 2005; Rajendran et al., 2008; Shimizu et al., 2008; Sannerud et al., 2011; Wolfe et al., 2013), we hypothesize that elevated levels of plasma LDL cholesterol disturb the structure and function of endolysosomes and promotes to the development of AD-like pathological features in skeletal muscle thus contributing to the skeletal muscle deficit observed in AD. Herein, we report that cholesterol-enriched diet induced morphological and functional changes of endolysosomes as well as abnormal accumulations of ubiquitin, phosphorylated tau, and Aβ/AβPP in skeletal muscle endolysosomes.

MATERIALS AND METHODS

Rabbits

New Zealand white female rabbits (1.5 to 2 years old) weighing 3 to 4 kg were fed either normal chow ($n = 5$) or a normal chow supplemented with 2% cholesterol ($n = 9$). After 12 weeks on the diet, animals were anesthetized and euthanized, and skeletal muscle (triceps) was dissected, frozen on liquid nitrogen cooled surface, and stored at −80°C until taken for experimentation. The animal protocol was approved by the University of North Dakota Animal Care and Use Committee adherent with the Guide for the Care and Use of Laboratory Animals (NIH publication number 80–23).

Immunohistochemistry

Cryostat-sectioned skeletal muscle (thickness 14 μm) was stained for target proteins using antibodies to EEA1 (Santa Cruz, sc-0415), LAMP2 (Santa Cruz, sc-8101), cathepsin D (Sigma, c0715), Aβ (4G8, Signet, 39220), phosphorylated tau (SMI-31, Covance, 141815001), ubiquitin (Santa Cruz, sc-8017), N-terminal AβPP (Chemicon, MAB348), C-terminal AβPP (Sigma, A8717), and microtubule-associated protein light chain 3 (LC3, Santa cruz, sc-16755). For immunohistochemistry for bright field microscopy, sections were developed with diaminobenzidine substrate using the avidin-biotin horseradish peroxidase system (Vector Laboratories) and counterstained with hematoxylin, and bright field images were taken by a Nikon Eclipse 80i (upright) microscope with a 40× PlanApo objective. Double fluorescence staining was used to determine co-localization of early endosome marker (EEA1, Abcam ab70521) with late endosome/lysosome marker (LAMP2, Santa Cruz, sc-8101) or autophagosome marker (LC3, Santa cruz, sc-16755) and subcellular co-distribution patterns of AβPP, Aβ, phosphorylated tau, and ubiquitin in early endosome EEA1 (Santa Cruz, sc-0415) and lysosomes (LAMP2, Santa Cruz, sc-8101) or autophagosomes (LC3, Santa cruz, sc-16755). Sections were examined by an Olympus FV300 laser scanning confocal microscope: Argon laser (488 nm, 10 mW) and HeNe laser (543 nm, 1 mW), external two-channel photomultiplier detection, FITC and Texas red probes, 60× oil PlanApo objective, Spot RT color CCD camera, and Fluoview software. Free cholesterol was stained with filipin (Sigma) and co-distribution of free cholesterol with endolysosomes was examined with a Leica DM4000B fluorescent microscope: 63× oil HCX PL Fluotar objective, DAPI and Texas red filters, Leica SCR camera, Leica Application Suite software. Images were process by Image J software or Photoshop CS5 software. Controls for specificity were used including staining muscle with an isotype-matched irrelevant antibody as a negative control, staining muscle with primary antibodies without

fluorescence-conjugated secondary antibodies (background controls), and staining muscle with only secondary antibodies – these controls allowed us to eliminate auto-fluorescence in each channel and bleed-through (crossover) between channels.

Immunoblotting

Skeletal muscle was homogenized mechanically in TPER extraction buffer (Pierce) at a ratio of 1:20 (w:v) in the presence of protease inhibitor cocktail (Sigma) and phosphatase inhibitors (5 mmol/l sodium fluoride and 50 μmol/l sodium orthovanadate). The detergent-soluble fraction (supernatant) was isolated by centrifugation at 100,000 × g for 1 h at 4°C. Protein concentration was determined by Bradford assay. Equal amounts of protein (100 μg) from detergent-soluble fractions were resolved by SDS-PAGE under reducing conditions, transferred to PVDF membranes, and subjected to immunoblotting with antibodies to N-terminal AβPP (1:1000, Chemicon, MAB348), phosphorylated tau (AT8, 1: 1000, Pierce, MN1020), tau 5 (1:1000, Calbiochem 577801), acid phosphatase (1:1000, Abcam, ab54720), cathepsin D (1:1000, Sigma, C0715), cathepsin B (1:1000, Sigma, C6243), Aβ (6E10, 1:500, Signet, 9320), and LC3 was detected using an anti-LC3 antibody (which recognizes both LC3-I and LC3-II, 1:500, Abcam, ab58610). Blots were probed with secondary antibodies conjugated with horseradish peroxidase (HRP) for 1 h at room temperature, reacted with luminal reagent, exposed, visualized, and analyzed by LabWorks 4.5 software on a UVP Bioimaging System (Upland). Quantification was performed by densitometry and the results were analyzed and normalized (vs. averaged densitometric volume values of control rabbits). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 1:5000, Abcam, ab8245) was used for loading controls.

Lysosomal Enzyme Activity Measurement

Acid phosphatase enzyme activity was determined using an Acid Phosphatase Assay kit (Sigma); a luminescence-based assay that uses 4-nitrophenyl phosphate as the substrate (Chen et al., 2010). Enzyme activities of cathepsin D and cathepsin B were determined using two separate assay kits (BioVision); fluorescence-based assays that use preferred MCA-labeled substrates for cathepsin-D and cathepsin B (Chen et al., 2010). Enzyme activities were expressed as relative opitcal density or fluorescence units (RFU) per 50 μg of total protein. Specific activities of each enzyme were indicated by the ratio of enzyme activity to protein levels as determined by immunoblotting

Statistical Analysis

All data were expressed as mean and SEM. Statistical significance was determined with unpaired two-tail Student's *t*-test (Frequentist tests indicated that all data were normally distributed). $P < 0.05$ was considered to be statistically significant.

RESULTS

Cholesterol-Enriched Diet Increases Accumulation of Cholesterol in Endolysosomes and Disturbs Endolysosome Structure and Function

Skeletal muscle has a very low capacity to synthesize cholesterol (Spady and Dietschy, 1983) and it meets its cholesterol needs mainly by taking up circulating lipoproteins. Extracellular cholesterol in the form of LDL is delivered into muscle cells via receptor-mediated endocytosis and transported to endolysosomes (Brown and Goldstein, 1986). In our cholesterol-fed rabbits, serum levels of cholesterol were about 10-times higher than normal (Chen et al., 2008b). Because LDL cholesterol is endocytosed in skeletal muscle, we examined the extent to which cholesterol was accumulated in endolysosomes. Using double fluorescence staining techniques with EEA1 as a marker for endosomes, filipin for free cholesterol, and LAMP2 for lysosomes, we found that filipin-positive staining of free cholesterol co-distributed with EEA1-positive endosomes (**Figure 1A**) and LAMP2-positive lysosomes (**Figure 1B**). None of these features were present in muscle from control rabbits. In addition, we found that EEA1-positive endosomes and LAMP2-positive lysosomes appeared to be enlarged (**Figures 1A,B**). To access further endolysosome morphological changes we stained for EEA1, LAMP2, and the lysosomal enzyme cathepsin D and found under light microscopy that endolysosome-positive signals (EEA1, LAMP2, and cathepsin D) were weak and diffuse in muscle from control rabbits, but endolysosome-positive staining was readily observed and appeared as large specifically stained aggregates in muscles from cholesterol-fed rabbits (**Figure 1C**). Furthermore, we found that the percentage of muscle fibers that contained such abnormally enlarged endolysosomes was 2.5 ± 0.6% (% to total muscle fibers examined). The percentage of impaired muscle fibers seems low, however, it is induced by cholesterol diet treatment for only 3 months. With longer treatment, we expect more muscle fibers will be affected, and this could have a significant impact for the pathogenesis of sporadic AD, which take decades to develop.

It is not clear how such abnormally enlarged endolysosomes are developed; however, based on recent findings that EEA1 positive early endosomes could fuse with lysosomes under disrupted endocytic homeostasis (Falcon-Perez et al., 2005; Das and Pellett, 2011; Ramanathan et al., 2013), the observed abnormally enlarged endolysosomes in cholesterol fed rabbits could result from uncontrolled fusion of endosomes with late endolysosome/lysosomes. As such, we determined the co-localization of EEA1 with LAMP2, and we found that EEA1 is co-localized with LAMP2 in those abnormally enlarged endolysosomes in muscle fibers from cholesterol-fed rabbits (**Figure 1D**). These findings suggest that increased cholesterol uptake and subsequent endolysosome accumulation of cholesterol in skeletal muscle could disrupt endocytic homeostasis and lead to uncontrolled fusion of early endosomes with late endosomes/lysosomes.

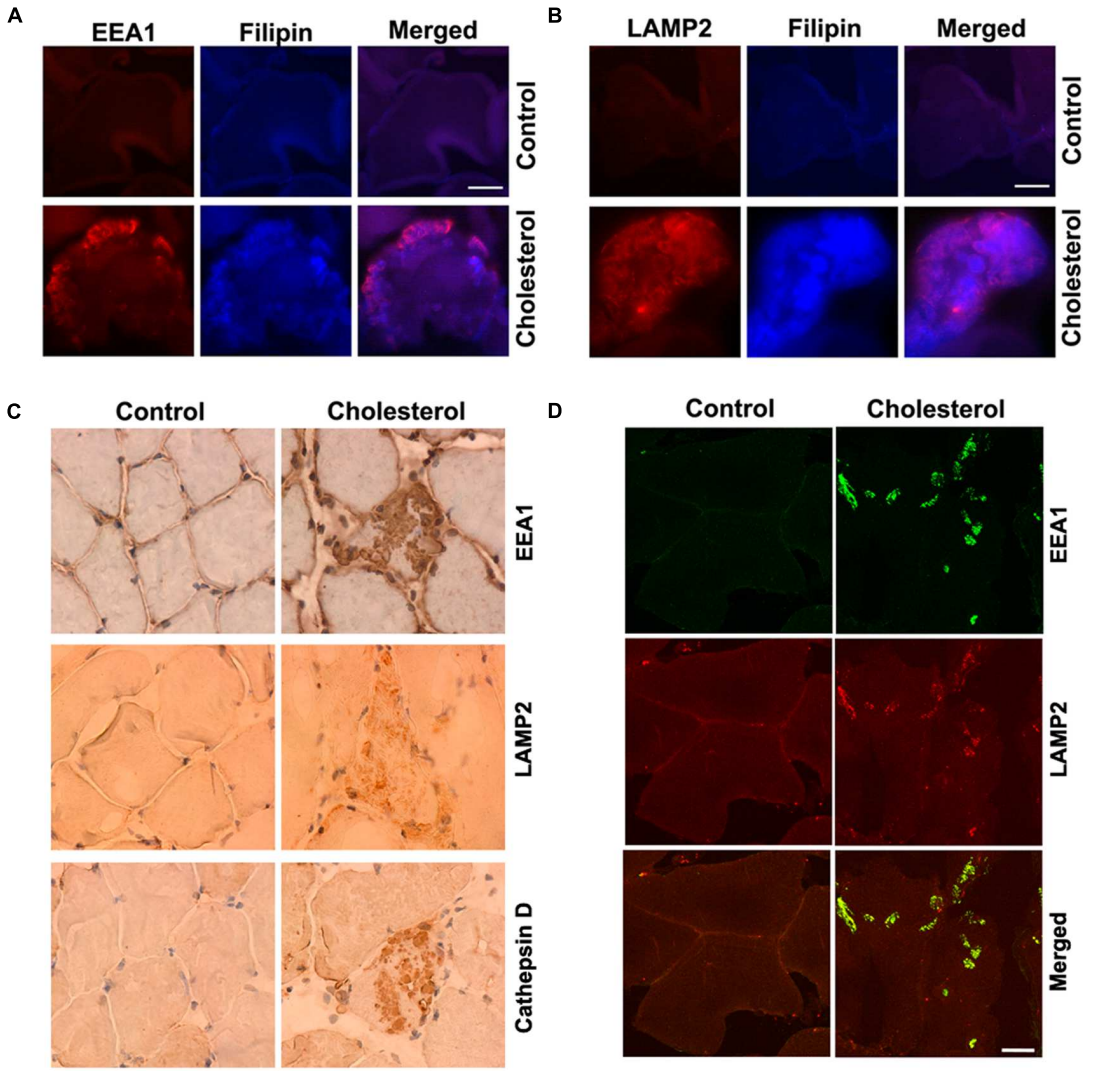

FIGURE 1 | Cholesterol-enriched diet increases accumulation of free cholesterol in endolysosomes and induces endolysosome enlargement. **(A,B)** Filipin-positive staining of free cholesterol (blue) co-distributed with EEA1-positive endosomes (red) and LAMP2-positive lysosomes (red) in muscle from cholesterol-fed rabbits. **(C)** In muscle fibers from control rabbits, EEA1, LAMP2, and cathepsin D staining appeared weak and diffuse, but in muscle fibers from cholesterol-fed rabbits, EEA1, LAMP2, and cathepsin D positive staining was strong forming large clumps (40X). **(D)** Cholesterol-enriched diet increased co-localization of EEA1 with LAMP2 in those abnormally enlarged endolysosomes. Bar = 20 μm.

Because of the above findings that cholesterol-enriched diet induced increases in cholesterol accumulation and endolysosome enlargement, we determined next the extent to which cholesterol-enriched diet affected endolysosome function by measuring protein levels and specific activities of three different enzymes; cathepsin D, cathepsin B, and acid phosphatase. We found that the cholesterol-enriched diet increased significantly protein levels of acid phosphatase (**Figure 2A**, $p < 0.05$), cathepsin D (**Figure 2C**, $p < 0.05$), and cathepsin B (**Figure 2E**, $p < 0.01$), but decreased significantly the specific enzyme activity (ratio of enzyme activity to protein levels) of acid phosphatase (**Figure 2B**, $p < 0.05$), cathepsin D (**Figure 2D**, $p < 0.05$), and cathepsin B (**Figure 2F**, $p < 0.01$). Together, these findings indicate that the cholesterol-enriched diet increased the accumulation of cholesterol in endolysosomes and altered the structure and function of endolysosomes in skeletal muscle. As a reminder, we would like to point out that such endolysosome changes are similar to those reported by others in brain neurons from AD patients (Cataldo and Nixon, 1990; Cataldo et al., 1994, 2000) and by us in brains of cholesterol-fed rabbits (Chen et al., 2010).

Cholesterol-Enriched Diet Increases the Accumulation of Phosphorylated tau, Ubiquitin, and AβPP in Endolysosomes

Multiple proteins that tend to aggregate including phosphorylated tau (Alonso et al., 1996; Alonso Adel et al., 2013; Wang et al., 2013), Aβ (Braak and Del Tredici, 2004;

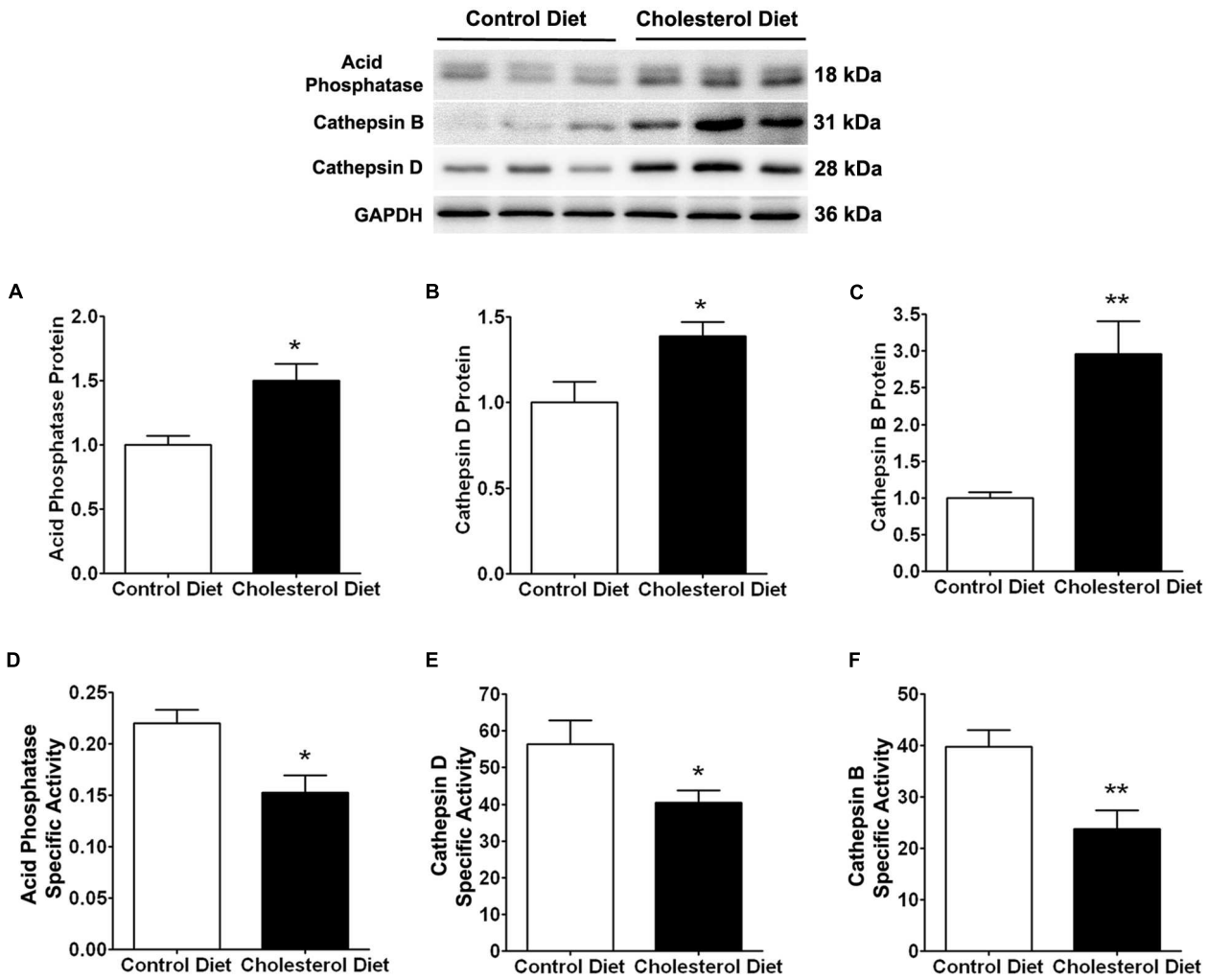

FIGURE 2 | Cholesterol-enriched diet impairs specific activities of endolysosome enzymes. Representative western blots were shown. The cholesterol-enriched diet ($n = 9$) increased significantly protein levels of acid phosphatase (**A**, $^*p < 0.05$), cathepsin D (**C**, $^*p < 0.05$), and cathepsin B (**E**, $^{**}p < 0.01$) in skeletal muscle, when compared with controls ($n = 5$). Cholesterol-enriched diet ($n = 9$) decreased significantly specific activities (ratio of total enzyme activity to protein levels as determined by immunoblotting) of acid phosphatase (**B**, $^*p < 0.05$), cathepsin D (**D**, $^*p < 0.05$), and cathepsin B (**F**, $^{**}p < 0.01$), when compared with controls ($n = 5$).

LaFerla et al., 2007), and ubiquitin (Dil Kuazi et al., 2003) are increasingly accumulated in neurons of individuals living with AD. We have shown that these proteins are accumulated in neurons of rabbits fed a cholesterol-enriched diet (Chen et al., 2010). More importantly, we showed previously that these same proteins were also deposited in skeletal muscle of rabbits fed the cholesterol-enriched diet (Chen et al., 2008b). In addition, all these proteins have been linked to endolysosomes; amyloidogenic processing of AβPP has been shown to occur mainly in endolysosomes following AβPP endocytosis (Nixon, 2005; Rajendran and Annaert, 2012; Morel et al., 2013; Jiang et al., 2014), ubiquitin has been shown to function as a signal for membrane protein internalization and protein degradation in the autophagy-lysosome system (Holler and Dikic, 2004; D'Agostino et al., 2011), and tau and phosphorylated tau have been reported to be degraded in the autophagy-lysosome system (Kenessey et al., 1997; Oyama et al., 1998; Hamano et al., 2008; Wang et al., 2009; Chesser et al., 2013). As described above, we demonstrated that cholesterol-fed rabbits markedly enlarged endolysosomes and disturbed their function in skeletal muscle. Thus, we determined next the extent to which cholesterol-enriched diet affected accumulation of these proteins in endolysosomes in skeletal muscle of rabbits fed a cholesterol-enriched diet.

To access endolysosome accumulation of tau protein, we stained phosphorylated tau and found under bright field microscopy that SMI-31 positive phosphorylated tau signals were weak in muscle from control rabbits, but SMI-31 positive phosphorylated tau signals was readily observed and appeared as large aggregates in muscles from cholesterol-fed rabbits (**Figure 3A**). The size and shape of these phosphorylated tau-positive signals were similar to those endolysosome-positive signals (**Figure 1C**), indicating the phosphorylated tau might

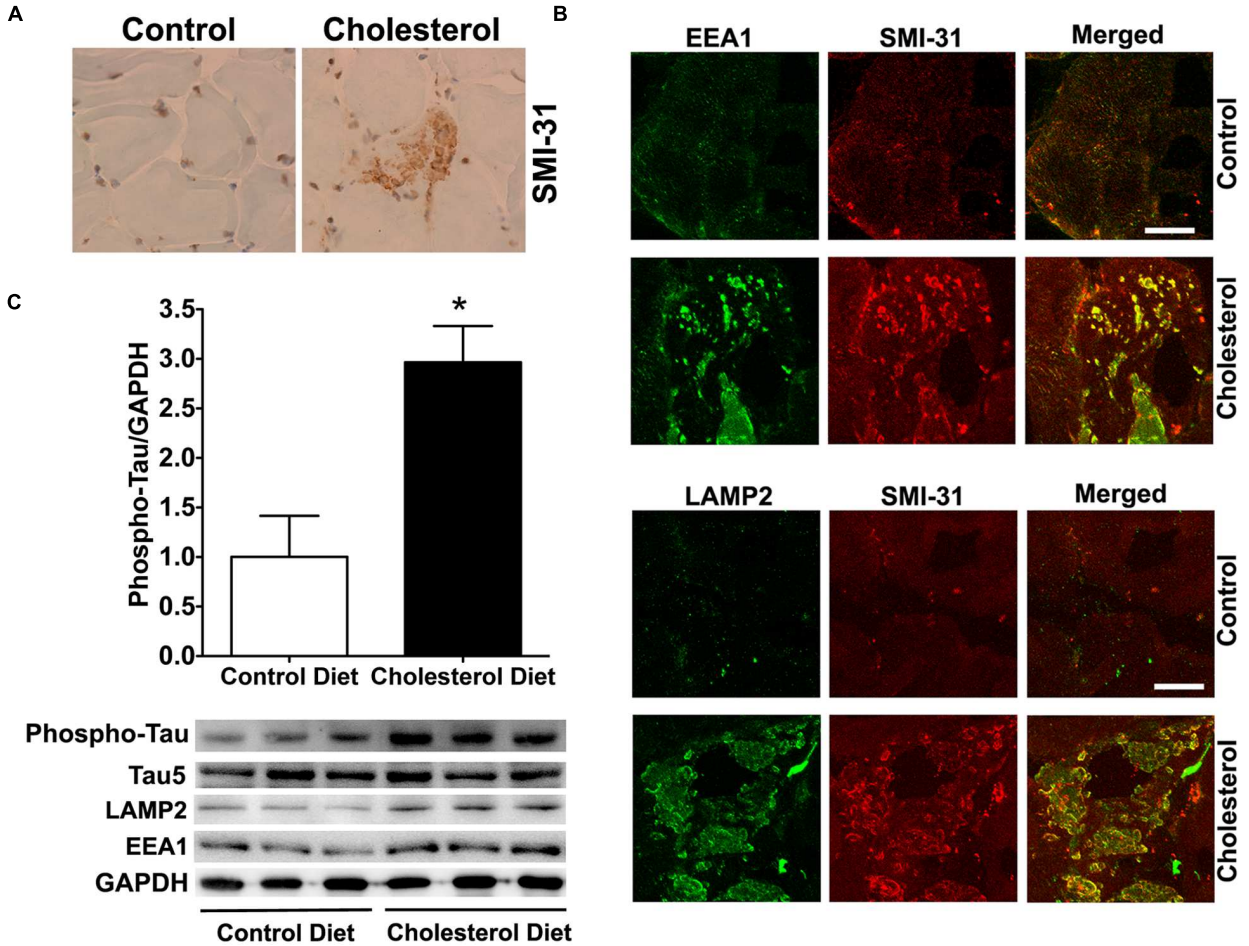

FIGURE 3 | Cholesterol-enriched diet increases the accumulation of phosphorylated tau in endolysosomes. (A) In muscle fibers from control rabbits, SMI-31 positive staining was weak, but in muscle fibers from cholesterol-fed rabbits SMI-31 positive staining appeared as large aggregates (40X). **(B)** SMI-31 positive staining of phosphorylated tau (red) co-distributed with EEA1-positive staining of endosomes (green) and LAMP2-positive staining of lysosomes (green) in muscle from cholesterol-fed rabbits. Bar = 20 μm. **(C)** Representative western blots were shown. Rabbits fed cholesterol-enriched diet ($n = 9$) exhibited significantly ($^*p < 0.05$) increased protein levels of phosphorylated tau in skeletal muscle, when compared with controls ($n = 5$).

be accumulating in abnormally enlarged endolysosomes. Using double fluorescence staining methods, we found that intramuscular depositions of phosphorylated tau were co-distributed with EEA1-positive endosomes and LAMP2-positive lysosomes (**Figure 3B**). Our findings are consistent with others' reports that tau and phosphorylated tau can be degraded in the autophagy-lysosome system (Kenessey et al., 1997; Oyama et al., 1998; Hamano et al., 2008; Wang et al., 2009; Chesser et al., 2013). Although tau and phosphorylated tau are not normally present in early endosomes, under conditions when endocytic homeostasis is disrupted with uncontrolled fusion of early endosomes with late endosomes/lysosomes (**Figure 1D**) it is possible for tau to be co-localized with EEA1 positive early endosomes. These morphological data suggest that cholesterol-enriched diet promotes the intramuscular accumulation of phosphorylated tau in abnormally enlarged endolysosomes. To extend and confirm further our observations, we examined next protein levels of phosphorylated tau using immunoblotting methods. We found significantly increased protein levels of phosphorylated tau (**Figure 3C**, $p < 0.05$) in skeletal muscle from cholesterol-fed rabbits when compared to those from control rabbits. Thus our data suggest that cholesterol-enriched diet leads to an increased accumulation of phosphorylated tau in abnormally enlarged endolysosomes in skeletal muscle.

To access endolysosome accumulation of ubiquitin, we stained ubiquitin and found under bright field microscopy that ubiquitin positive signals were weak in muscle from control rabbits, but ubiquitin positive staining was readily observed and appeared as large aggregates in muscles from cholesterol-fed rabbits (**Figure 4A**). The size and shape of these ubiquitin positive signals were also similar to those endolysosome-positive signals (**Figure 1C**), indicating the ubiquitin might be accumulating in abnormally enlarged endolysosomes. Using double fluorescence staining methods, we found that intramuscular depositions of ubiquitin were co-distributed with EEA1-positive endosomes and LAMP2-positive lysosomes (**Figure 4B**).

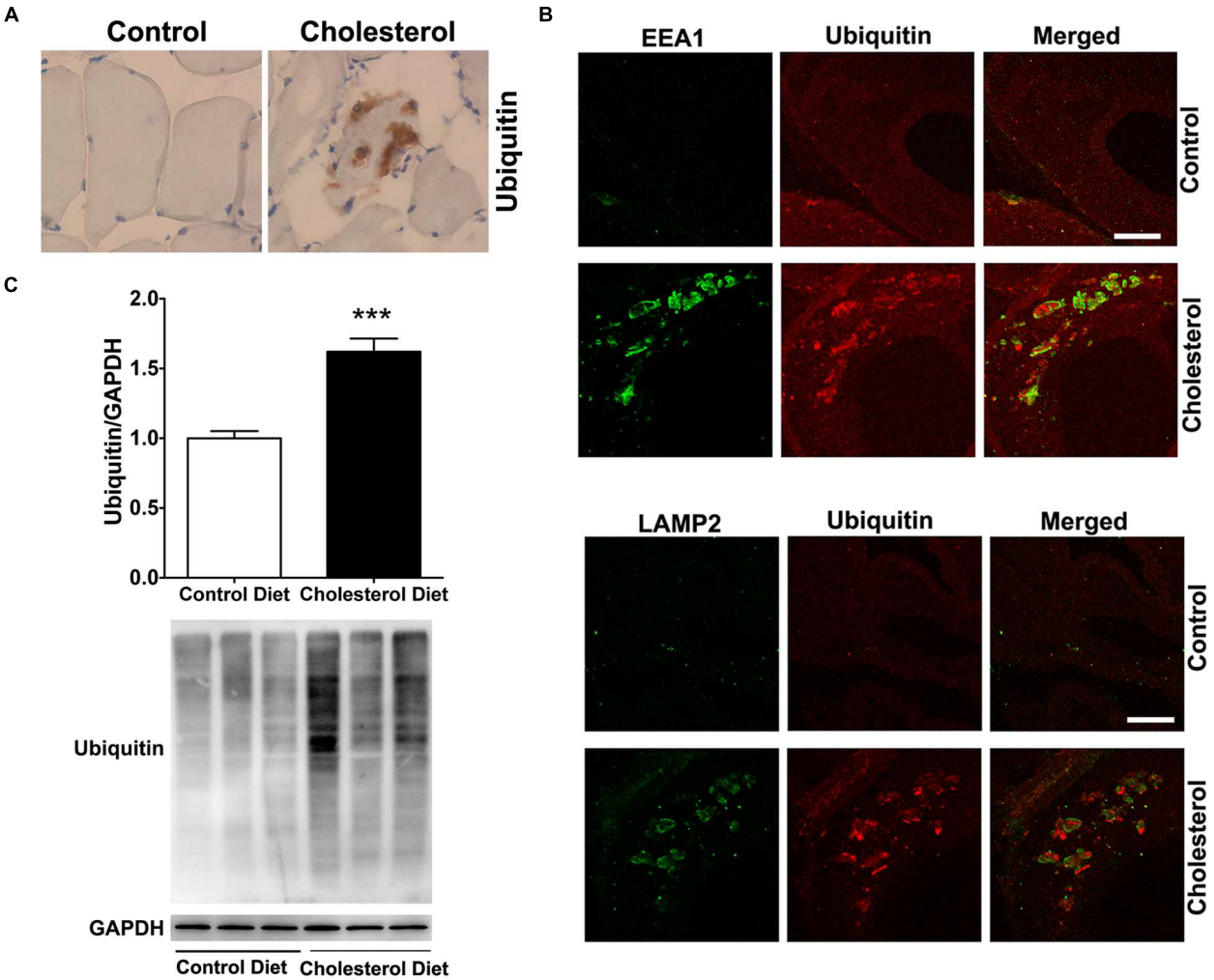

FIGURE 4 | Cholesterol-enriched diet increases the accumulation of ubiquitin in endolysosomes. (A) In muscle fibers from control rabbits, ubiquitin positive staining was weak, but in muscle fibers from cholesterol-fed rabbits ubiquitin positive staining appeared as large aggregates (40X). **(B)** Ubiquitin positive staining (red) co-distributed with EEA1-positive staining of endosomes (green) and with LAMP2-positive staining of lysosomes (green) in muscle from cholesterol-fed rabbits. Bar = 20 μm. **(C)** Representative western blots were shown. Rabbits fed cholesterol-enriched diet ($n = 9$) exhibited significantly (***$P < 0.001$) increased protein levels of ubiquitin in skeletal muscle, when compared with controls ($n = 5$).

These morphological data suggest that ubiquitin accumulates intramuscularly in abnormally enlarged endolysosomes, which are consistent with others' reports that ubiquitin functions as a signal for membrane protein internalization and protein degradation in the autophagy-lysosome system (Holler and Dikic, 2004; D'Agostino et al., 2011). To extend and confirm further our observations, we examined next protein levels of ubiquitin using immunoblotting methods. We found significantly increased protein levels of ubiquitin (**Figure 4C**, $p < 0.001$) in skeletal muscle from cholesterol-fed rabbits when compared to those from control rabbits. Thus, our data suggest that cholesterol-enriched diet leads to an increased accumulation of ubiquitin in abnormally enlarged endolysosomes in skeletal muscle. The presence of ubiquitin positive inclusions indicate a general degeneration process is occurring. To assess whether such general degeneration might be involved in the pathogenesis of Parkinson's disease or amyotrophic lateral sclerosis, we determined the expression of α-synuclein and TPD-43 using immunohistochemistry. We found positive but weak immunopositive staining for α-synuclein and TPD-43 in impaired muscle fibers (Supplementary Data). These findings are consistent with our findings that cholesterol-enriched diet induces a general degeneration process as evidenced by the presence of ubiquitin positive inclusions. However, given the weak staining signals for α-synuclein and TPD-43, it is not likely that cholesterol-enriched diet plays a significant or specific pathological role in the development of Parkinson's disease or amyotrophic lateral sclerosis.

To access endolysosome accumulation of AβPP protein, we stained AβPP with a N-terminal AβPP antibody or a C-terminal AβPP antibody (data not shown) and found under bright field microscopy that AβPP positive signals were mainly present

at plasma membrane and intramuscular signals were weak in muscle from control rabbits, but AβPP positive signals were readily observed and appeared as large aggregates inside muscles from cholesterol-fed rabbits (**Figure 5A**). The size and shape of these AβPP positive signals were also similar to those endolysosome-positive signals (**Figure 1C**), indicating the AβPP might be accumulating in abnormally enlarged endolysosomes. Using double fluorescence staining methods, we found that intramuscular depositions of AβPP were co-distributed with EEA1-positive endosomes and LAMP2-positive lysosomes (**Figure 5B**). These morphological data suggest that AβPP accumulates intramuscularly in abnormally enlarged endolysosomes. To extend and confirm further our observations, we examined next protein levels of AβPP using immunoblotting methods. We found significantly increased protein levels of AβPP (**Figure 5C**, $p < 0.01$) in skeletal muscle from cholesterol-fed rabbits when compared to those from control rabbits. Thus, our data suggest that cholesterol-enriched diet leads to an increased accumulation of AβPP in abnormally enlarged endolysosomes in skeletal muscle.

Aβ Accumulates in Endosomes and Autophagosomes in Skeletal Muscle of Rabbits Fed Cholesterol-Enriched Diet

Endolysosomes play a critical role in amyloidogenic processing of AβPP (Rajendran and Annaert, 2012; Morel et al., 2013; Jiang et al., 2014) and they are major sites where Aβ is generated following internalization of AβPP (Nixon, 2005; Rajendran et al., 2008; Shimizu et al., 2008; Sannerud et al., 2011; Rajendran and Annaert, 2012; Morel et al., 2013; Jiang et al., 2014). Once generated, Aβ can be degraded by cathepsins in lysosomes (Miners et al., 2011) and the remaining Aβ can either accumulate in endolysosomes where it may precipitate AD pathogenesis (Braak and Del Tredici, 2004; LaFerla et al., 2007; Zou et al., 2015) or it can be released via exocytosis (Annunziata et al., 2013; Nilsson et al., 2013). Accordingly, Aβ levels can be enhanced by those factors that promote amyloidogenic processing of AβPP (Grbovic et al., 2003; Ma et al., 2009) and those that impair Aβ degradation (Torres et al., 2012).

Given our findings that cholesterol enriched diet increases cholesterol accumulation in endolysosomes and dramatically

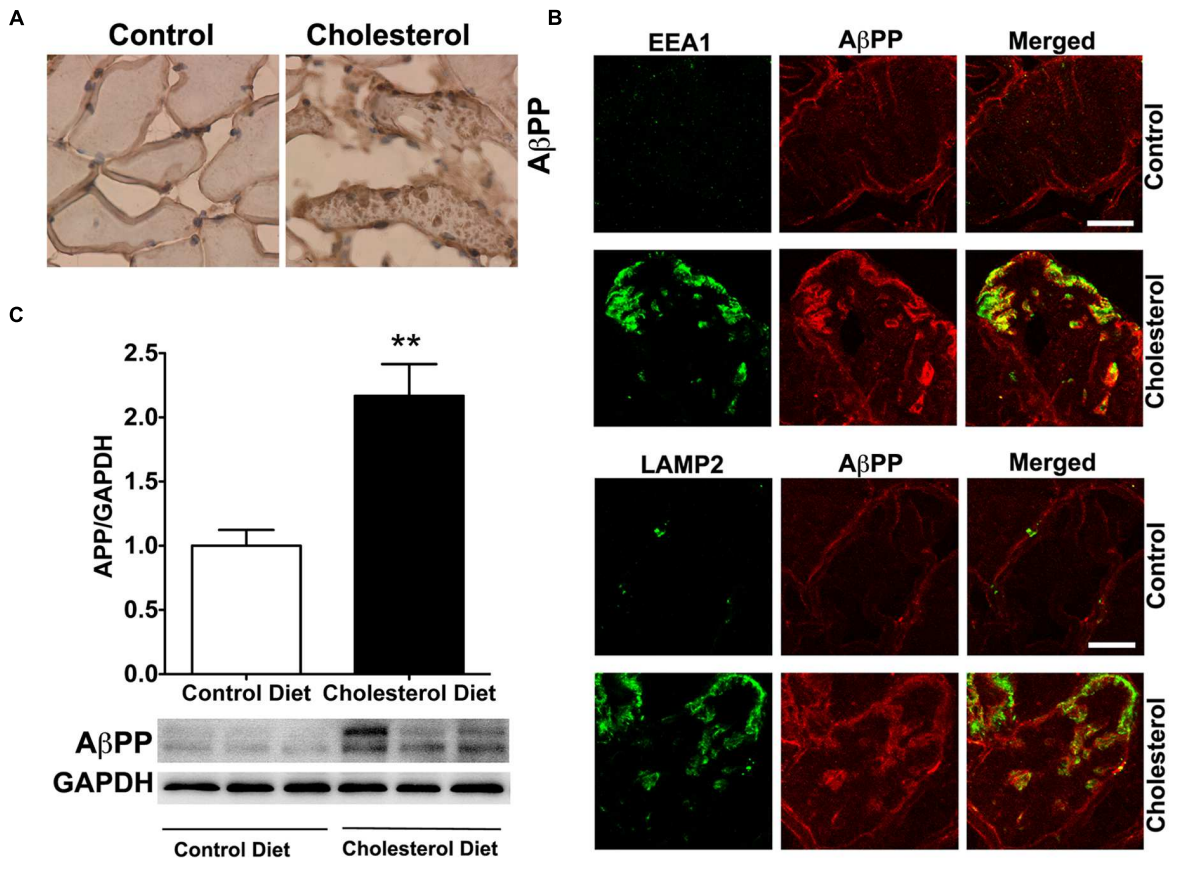

FIGURE 5 | Cholesterol-enriched diet increases the accumulation of AβPP in endolysosomes. (A) In muscle fibers from control rabbits, N-terminal AβPP positive signals were mainly present at plasma membrane and intramuscular signals were weak, but in muscles from cholesterol-fed rabbits AβPP positive signals appeared as large aggregates inside muscles (40X). **(B)** N-terminal AβPP positive staining (red) co-distributed with EEA1-positive staining of endosomes (green) and with LAMP2-positive staining of lysosomes (green) in muscle from cholesterol-fed rabbits. Bar = 20 μm. **(C)** Representative western blots were shown. Rabbits fed cholesterol-enriched diet ($n = 9$) exhibited significantly (**$P < 0.01$) increased protein levels of AβPP in skeletal muscle, when compared with controls ($n = 5$).

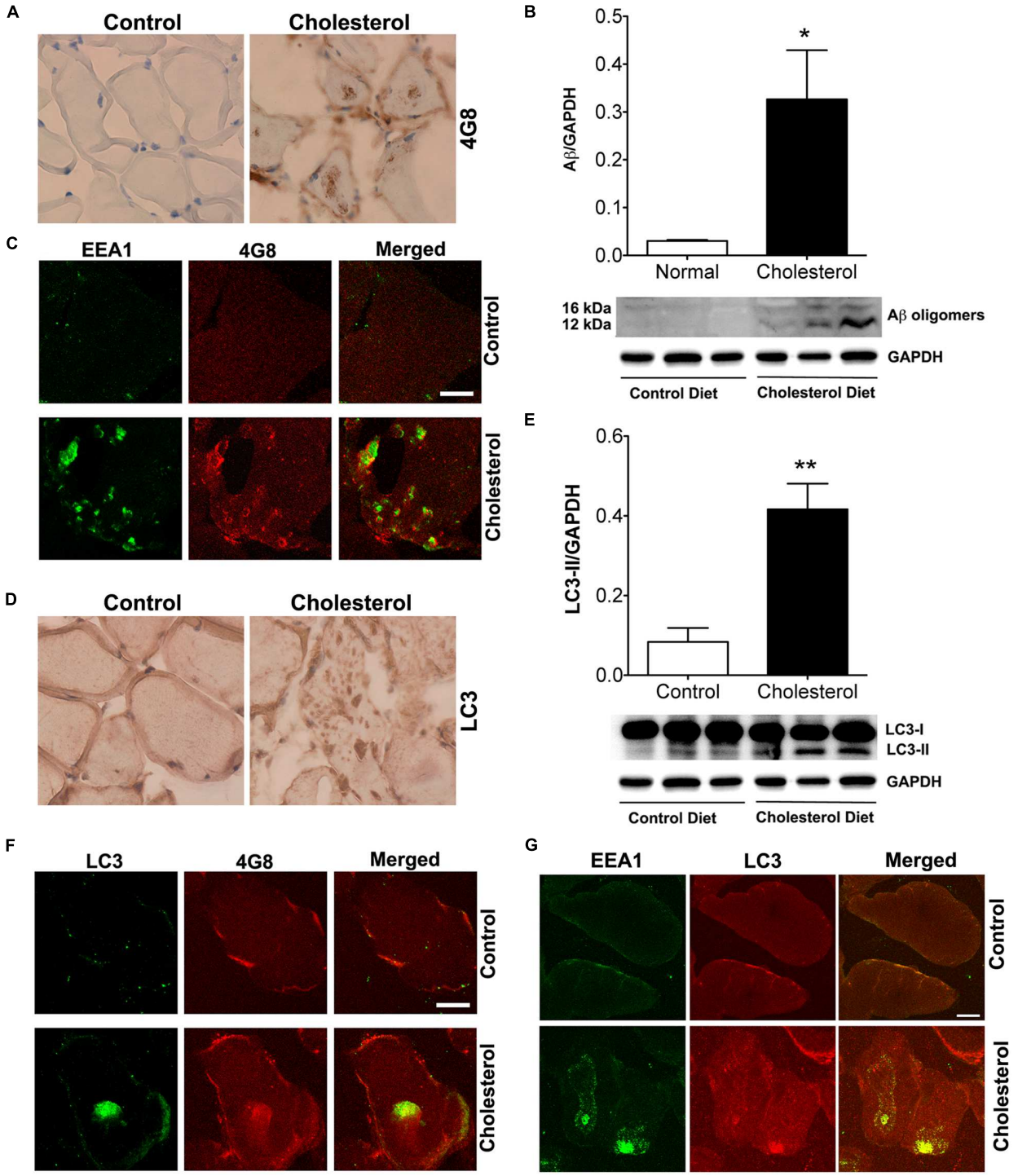

FIGURE 6 | Cholesterol-enriched diet increases deposition of Aβ in endosomes and autophagosomes. (A) In muscle fibers from control rabbits, 4G8 positive Aβ signals were weak, but in muscles from cholesterol-fed rabbits 4G8 positive Aβ signals appeared as large aggregates inside muscles (40X). **(B)** Representative western blots were shown. Cholesterol-enriched diet ($n = 9$) increased significantly protein levels of Aβ oligomers ($^*p < 0.05$) in skeletal muscle, when compared with controls ($n = 5$). **(C)** 4G8-positive staining of Aβ (red) co-distributed with EEA1 positive staining of endosomes (green) in skeletal muscle from cholesterol-fed rabbits. Bar = 20 μm. **(D)** In muscle fibers from control rabbits, LC3 positive autophagosome signals were weak and diffuse, but appeared as large aggregates inside muscles from cholesterol-fed rabbits (40X). **(E)** Representative western blots were shown. Cholesterol-enriched diet ($n = 9$) increased significantly protein levels of LC3-II ($^{**}p < 0.01$) in skeletal muscle, when compared with controls ($n = 5$). **(F)** 4G8-positive staining of Aβ (red) co-distributed with LC3-positive staining for autophagosomes (green) in skeletal muscle from cholesterol-fed rabbits. Bar = 20 μm. **(G)** EEA1positive endosomes (green) co-distributed with LC3-positive staining for autophagosomes (red) in skeletal muscle from cholesterol-fed rabbits. Bar = 20 μm.

altered structure and function of endolysosomes, we determined the extent to which cholesterol-enriched diet affects levels of intramuscular Aβ. Using the 4G8 antibody for detection of Aβ, we found under bright field microscopy that 4G8 positive Aβ signals appeared as large aggregates inside muscles from cholesterol-fed rabbits (**Figure 6A**). Using immunoblotting methods, we found that cholesterol-enriched diet increased significantly protein levels of Aβ oligomers (**Figure 6B**, $p < 0.05$) in skeletal muscle from cholesterol-fed rabbits. Using double fluorescent staining, we found that 4G8-positive Aβ staining co-distributed with EEA1-positive endosomes (**Figure 6C**), a finding consistent with endosome production of Aβ. None of these features was present in skeletal muscle of control rabbits. Our observations of increased accumulation of Aβ/AβPP in endolysosomes suggests to us that increased amyloidogenic processing of AβPP was caused by morphological and functional alterations in endolysosomes.

Because Aβ was also found to be a substrate for autophagy (Nixon, 2007), we next determined the extent to which Aβ accumulated in autophagosomes. We found under light microscopy that LC3 positive autophagosome signals were weak and diffuse in muscles from control rabbits, but appeared as large aggregates inside muscles from cholesterol-fed rabbits (**Figure 6D**). Using immunoblotting methods, we found that cholesterol-enriched diet increased significantly protein levels of LC3-II, an indicator of autophagosome proliferation and maturation (**Figure 6E**, $p < 0.01$). Using double fluorescent staining, we found that 4G8-positive Aβ staining co-distributed with LC3 positive autophagosomes (**Figure 6F**). The observation that Aβ is present in LC3-positive autophagosomes suggests further the presence of dysfunctional endolysosome-autophagosome system. Given our finding that cholesterol-enriched diet increases the co-localization of EEA1 with LAMP2 (**Figure 1D**), which indicates endolysosome accumulation of cholesterol could lead to uncontrolled fusion of early endosomes with late endosome/lysosomes, we determined the extent to which cholesterol-enriched diet affects the co-localization of EEA1 with LC3. We found that cholesterol-enriched diet increased dramatically co-localization of EEA1 with LC3 (**Figure 6G**). These observations indicate that endolysosome accumulation of cholesterol could also increase fusion of early endosomes with autophagosome, a phenomenon that fosters maturation of autophagosomes (Tooze and Razi, 2009).

DISCUSSION

The present studies were aimed to test the hypothesis that endolysosome dysfunction in skeletal muscle shares common pathological features to those found in brain in a rabbit model of sporadic AD. Principally, we demonstrated that skeletal muscle from a cholesterol-fed rabbit model of AD exhibited increased cholesterol accumulation in endolysosomes, enlarged endolysosomes, and impaired endolysosome function. Furthermore, we demonstrated that various AD marker proteins including phosphorylated tau, ubiquitin, AβPP and Aβ were accumulated in those enlarged endolysosomes. Together these findings provide further insight into skeletal muscle involvement in AD and the potentially significant role that endolysosomes play in the pathogenesis of AD.

Endolysosomes are acidic organelles consisting of endosomes, lysosomes, and autophagosomes that play a key role in protein turnover and cellular homeostasis (Appelqvist et al., 2013). Substrates for degradation are delivered to lysosomes by two general routes namely endocytosis and autophagy. Endocytosis is responsible for up-taking extracellular nutrients as well as turnover of plasma membrane proteins. Autophagy, on the other hand, is responsible for removing intracellular protein aggregates and "worn out" organelles. Endolysosomes are especially important for physiological functions of neurons and skeletal muscle cells because they are long-lived post-mitotic cells that require efficient endolysosomes to degrade intracellular protein aggregates, eliminate "worn out" organelles and maintain membrane integrity. As such, endolysosome dysfunction in neurons has been linked to neurodegeneration and has been shown to play an early and important role in the development of AD (Cataldo et al., 2000; Nixon, 2005; Rajendran et al., 2008; Shimizu et al., 2008; Sannerud et al., 2011). Similarly, endolysosome dysfunction in skeletal muscle contributes to muscle degeneration and age-related disorders and has been implicated in a variety of autophagosome and lysosome related myopathies (Askanas et al., 2009; Nogalska et al., 2010; Malicdan and Nishino, 2012; Bonaldo and Sandri, 2013; Demontis et al., 2013; Sandri et al., 2013). Interestingly, skeletal muscle deficits such as loss of muscle mass and reduced muscle strength has been shown to be early signs of AD that contribute to disability and could predict the onset and progression of clinical AD (Buchman et al., 2007; Boyle et al., 2009; Burns et al., 2010). However, the question of whether endolysosome dysfunction leads to development of pathological features of AD in skeletal muscle and contributes to skeletal muscle deficits in AD has not been addressed. As such, we determined the extent to which elevated levels of plasma LDL cholesterol, a robust risk factor for AD, disturbed the structure and function of endolysosomes and promoted the development of AD-like pathological features including intracellular deposition of Aβ/AβPP, phosphorylated tau, and ubiquitin in skeletal muscle.

Given that plasma lipoproteins supply skeletal muscle with needed cholesterol (Spady and Dietschy, 1983), elevated plasma LDL cholesterol and subsequent increases in LDL cholesterol uptake could lead to increased cholesterol accumulation in endolysosomes and disturbed endolysosome structure and dysfunction. As expected, we demonstrated cholesterol-enriched diet increased free cholesterol in endosomes and lysosomes in skeletal muscle. Importantly, we demonstrated that cholesterol-enriched diet dramatically enlarged endolysosomes and inhibited endolysosome function. Our observations that cholesterol-enriched diet increased co-localization of EEA1 with LAMP2 and LC3 indicate that abnormally enlarged endolysosomes might result from uncontrolled fusion of early endosomes

with late endosome/lysosomes or autophagosomes, a finding that is consistent with recent reports that EEA1 positive early endosomes could fuse with lysosomes under disrupted endocytic homeostasis (Falcon-Perez et al., 2005; Das and Pellett, 2011; Ramanathan et al., 2013).

The observations that cholesterol enriched diet increased accumulation of Aβ/AβPP in endolysosomes suggest to us that increased amyloidogenic processing of AβPP was caused by morphological and functional alterations in endolysosomes. The observation that Aβ is also present in LC3-positive autophagosomes suggests further the presence of dysfunctional endolysosomes because autophagic activity itself is part of the endolysosome system. Elevated levels of LDL cholesterol could promote Aβ deposition in skeletal muscle in two ways. First, receptor-mediated endocytosis of LDL cholesterol could encourage the accumulation of AβPP in endolysosomes by either promoting AβPP internalization (Waldron et al., 2006, 2008; Klug et al., 2011) or impairing the recycling of internalized AβPP back to plasma membrane (Andersen et al., 2005; Willnow and Andersen, 2013). Second, endolysosome inhibition could lead to impairment of Aβ degradation in lysosomes because Aβ can be degraded in lysosomes by cathepsin D and B (Nogalska et al., 2012; Saido and Leissring, 2012). Our observations that cholesterol-enriched diet increases endolysosome accumulation of AβPP and decreased specific enzyme activities of cathepsin D and B support, at least indirectly, both possible mechanisms.

The observations that cholesterol-enriched diet increased accumulation of ubiquitin and phosphorylated tau in endolysosomes in skeletal muscle indicate that endolysosome dysfunction as induced by a cholesterol-enriched diet contributes to the development of AD-like ubiquitin-positive multi-protein aggregates and inclusions in skeletal muscle. These findings are consistent with other's reports that increased accumulation of cholesterol in lysosomes and subsequent lysosome dysfunction has been linked to the development of neurofibrillary tangles in brains of patients with Niemann-Pick type C disease (Sawamura et al., 2001; Bu et al., 2002; Distl et al., 2003; Vance, 2006; Bi and Liao, 2007; Liao et al., 2007) and our reports that endolysosome dysfunction as induced by high levels of LDL cholesterol contributes to the development of tau-pathology in neurons (Chen et al., 2010; Hui et al., 2012).

Although a causal relationship is not determined, our findings do suggest that endolysosome dysfunction as induced by elevated plasma LDL cholesterol, aging, or other factors may play a pathogenic role in the development of AD-like pathological changes observed in skeletal muscle, similar to its role in neurodegeneration and the pathogenesis of AD in brain. Furthermore, our findings support the notion that common pathogenic mechanisms may exist in skeletal muscle and neurons, and that the skeletal muscle changes may represent early and progressive pathological features of AD.

AUTHOR CONTRIBUTIONS

XC, OG, and JG contributed to design the work. XC and JW contributed to the acquisition and analysis of data. XC drafted the work. XC, JW, OG, and JG contributed to interpretation of data and revising the work of intellectual content and final approval of the version to be published. XC drafted the work. XC, JW, OG, and JG approved of the final version and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fnagi.2016.00129

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OPINION
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Camptocormia in Parkinson's Disease: A Muscle Disease Due to Dysregulated Proprioceptive Polysynaptic Reflex Arch

Walter J. Schulz-Schaeffer *

Prion and Dementia Research Unit, Department of Neuropathology, University Medical Center Göttingen, Göttingen, Germany

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Camptocormia (from the Greek "kamptein" = to bend and "kormos" = trunk) is an anterior flexion of the thoracolumbar spine while standing, walking, or sitting that disappears in the supine position. The syndrome, also known as "bent spine syndrome," occurs in nearly 10% of idiopathic Parkinson's disease (iPD) patients (Yoritaka et al., 2013), but also in other neurodegenerative diseases and may occur in myopathies with axial involvement, in all forms of myositis, in dystonia, as a pharmacological side effect or as functional disorder. It causes marked impairment in quality of life and often leads to social isolation. The pathophysiology of camptocormia is as heterogenous as the causes of underlying diseases are, but recent work has provided some insights into the pathophysiology of PD-associated camptocormia and opened options for treatement. These findings show, how important it is to understand the interaction between muscle innervation, Golgi tendon organ and the central nervous system in regulating muscle tone.

DYSTONIA vs. MYOPATHY

Although, the bent spine syndrome has been known since first descriptions by Earle (1815) and appears in the original description of James Parkinson's case series in 1817 (Parkinson, 2002), the association of camptocormia with Parkinson's disease was definitively described by Djaldetti et al. (1999). Subsequently several small case series including muscle biopsies have been published, and myositis, mitochondrial disturbances, dystonia, or myopathy have been discussed as etiologies of the syndrome.

In the beginning of this century, larger series of paraspinal muscle biopsies in iPD patiens suffering from camptocormia revealed myopathic changes (Margraf et al., 2010; Spuler et al., 2010). Subsequently it was possible to exclude a mitochondriopathy and to define the myopathological changes that were common in all iPD camptocormia patients. By comparing samples of paraspinal and deltoid (a common biopsy muscle of the limbs) muscles of myopathologically healthy autopsy controls, a higher physiological content of mitochondria and so called "ragged red fibers" in the paraspinal than in the limb muscles was observed (Wrede et al., 2012), showing that mitochondrial content in muscle fibers seems to depend on dedication, force and function of muscles. By comparing the mitochondrial pattern in paraspinal muscles of 14 camptocormia biopsies and 10 autopsy controls, a mitochondriopathy in camptocormia of iPD patients could be ruled out (Wrede et al., 2012).

Interestingly, in the paraspinal muscles of all iPD camptocormia patients a reduction in type-2 fibers were observed, as well as a marked increase in size of type-1 fibers, an increase in connective tissue and fatty degeneration within muscle fascicles, and frequently defects located centrally in muscle fibers, comprising a loss of oxidative enzymes, visible in NADH-TR, SDH, COX, and MAG reactions. Within these defects, an increase of acid phosphatase reactivity was observed. Ultrastructurally, the defects showed a disarrangement of contractile elements and rod-like structures (Wrede et al., 2012). A myositis was not detectable. The lesions are different

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Luciano Merlini, Istituto Ortopedico Rizzoli, IRCCS, Italy

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Caroline Sewry, Great Ormond Street Hospital, UK
Marie-Bernadette Delisle, Toulouse III University - Paul Sabatier, France

***Correspondence:**
Walter J. Schulz-Schaeffer
wjschulz@med.uni-goettingen.de

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from central core diseases, although a ryanodine receptor 1 mutation can cause a camptocormia (Loseth et al., 2013). In core diseases, a type-1 fiber hypotrophy is seen and lesions lack acid phosphatase reactivity (Jungbluth, 2007).

The interpretation of these changes suggests a myopathy that could explain the loss of muscle strength in the paraspinal muscles as the main clinical symptome of camptocormia. Whereas all key aspects of the myopathological changes were detectable in all camptocormia muscle biopsies, the extent varied. Especially the extent of (reactive) fibrosis and (reactive) fatty degeneration of paraspinal muscles differed. The degree of fibrosis correlate with the severity of the syndrome (Wrede et al., 2012). Biopsies of muscles involved in dystonic movements have up to now revealed no histopathologically detectable myopathological changes (Swash and Fox, 1976). This may be because dystonic events result in transient muscle contraction, whereas clinical investigations in camptocormia suggest continuous muscle contraction. A hardening of paraspinal muscles is always found when iPD camptocormia patients are in an upright position and electromyographical recordings show continuously elevated firing (DiMatteo et al., 2011; Doherty et al., 2011; Tinazzi et al., 2013). Myopathologically, camptocormia is unlikely due to a dystonia, but the myopathological changes may be due to a secondary myopathy. Fibrosis and fatty changes are obviously secondary.

MYOPATHOLOGICAL FINDINGS IN CAMPTOCORMIA LEAD TO THE HYPOTHESIS OF PROPRIOCEPTIVE DYSREGULATION

The myopathological findings in camptocormia show remarkable parallels to those that were found 40 years ago by Karpati et al. in experimental tenotomy of achilles tendons in rats. Experimental tenotomy leads to core-like lesions in the center of type-1 fibers, which show an increase in acid phosphatase activity but not an increase in lysosomal structures (Karpati et al., 1972). Fibers of tenotomised muscles lack ATPase and SDH activity (Shafiq et al., 1969). Ultrastructurally, the core-like lesions have no normal register of sarcomeres. Sarcomeres in the lesions were disintegrated and Z-band streaming and electron-dense patches or plaques could be observed. In the lesions, mitochondria were reduced (Shafiq et al., 1969). These changes could not be observed in muscles that were tenotomized after denervation or after chordotomy (Karpati et al., 1972). Obviously, these experimental lesions developed only after interruption of the muscle tension input to the polysynaptic reflex arch, while the rest of the reflex arch was functionally intact. The description depicts the situation of a proprioceptive dysregulation.

The lesions described in tenotomy experiments are strikingly similar to the lesions observed in paraspinal muscles of camptocormia patients. These similarities point to the likelihood that camptocormia may also be related to a proprioceptive dysregulation. Other than in experimental tenotomy, the lesion site in camptocormia is most likely not at the level of the muscles. The association of camptocormia with Parkinson's disease suggests that the lesion is at the level of the central nervous system. Muscle tone regulation of paraspinal muscles is different from that of limb muscles (Gurfinkel et al., 2006). The body mass is anterior to the spinal column and the function of axial (i.e., paraspinal) muscles is to compensate any head or limb movements immediately to stabilize the trunk. Whereas, in limb muscles a shortening of the muscle length is associated with an increase in muscle tone, in paraspinal muscles the tonic level needs to increase in stretched muscles in order to stabilize the axis (Wright et al., 2007). The basal ganglia are involved in the control of postural muscle tone via proprioception (Takakusaki et al., 2004; Konczak et al., 2009). In PD, axial muscles are hypertonic and the hypertonicity correlates with UPDRS (unified Parkinson disease rating scale) scores (Wright et al., 2007). A deficit in the integration of proprioceptive information in postural control has been shown in PD (Vaugoyeau et al., 2011; Mongeon et al., 2015). It is possible that camptocormia in PD is an extreme form of muscle tone dysregulation.

PARALLELS BETWEEN CAMPTOCORMIA AND TENDON RUPTURE SEEN WITH IMAGING METHODS

Clinical presentation and MR images of the paraspinal muscles in camptocormia show remarkable parallels with the cramp-like pain and radiological alterations seen with torn tendon muscles. In camptocormia, the syndrome presents with serious back pain and a marked, well-palpable hardening of paraspinal muscles (Margraf et al., 2010). MR imaging shows a hyperintense signal of paraspinal muscles in the STIR sequence and a signal decrease in T1-weighted images, indicating edema and swelling. In other camptocormia patients, however, an increase in signal intensity in T1-weighted images has been observed. This is interpreted as fatty degeneration and muscle atrophy. Interestingly, these changes are associated with the length of time that patients suffered from camptocormia (Nakane et al., 2015). Whereas, edema and swelling are observed in the first 2 years of the syndrome, fatty degeneration and atrophy are observed later in the disease course (Margraf et al., 2015). With torn tendons, an initial painful contraction in the respective muscles can be observed, as for example in "Popeye syndrome," when the biceps is involved (Delle Rose et al., 2012). Subsequently, structural alterations in the muscle and muscle shrinkage will take place. These alterations can be visualized by CT and MR imaging. The Goutallier classification in radiology provides stages of deterioration (Goutallier et al., 1994). Higher stages (more advanced deterioration) were explained by progressing fibrosis and fatty degeneration of the involved muscles (Gerber et al., 2009; Hoffmann et al., 2011).

OTHER REASONS FOR MYOPATHOLOGICAL ALTERATIONS IN PARASPINAL MUSCLES THAT RESEMBLE THOSE OF CAMPTOCORMIA

The pattern of acid phosphatase-reactive myofibrillar disarrangement of paraspinal muscles is not limited to

camptocormia. Similar myopathological changes may be observed in disc herniation, scoliosis, or in aged individuals (Mattila et al., 1986; Wharton et al., 1996), but not all these patients develop camptocormia-like myopathological changes (Ford et al., 1983). In general, the fibrosis is less severe in patients suffering from disk herniation than in camptocormia patients (Delisle et al., 1993). It is most likely that the myofibrillar disarrangement of paraspinal muscles can be induced by compression of nerve root fibers or dorsal root ganglia. Causes of compression may be herniation of an intervertebral disc or bony changes such as spondylophytes of vertebrae, a phenomenon that is frequently observed in older persons, or malformation of vertebrae. It seems to be irrelevant for the development of the characteristic myopathological changes, whether the disturbances of the proprioceptive polysynaptic reflex arch occur at the level of the Golgi tendon organ (tension receptor), the dorsal root ganglia (afferent fibers of tendon organs and muscle spindles), or of the central control. A synopsis of the different pathways is provided in **Figure 1**.

EFFECT OF STN NEUROSTIMULATION ON CAMPTOCORMIA IN PD SUGGESTS CENTRAL MODULATION OF PROPRIOCEPTION

The hypothesis that defects in the central control of proprioceptive function underlie the pathophysiology of camptocormia has led to the notion that deep brain stimulation (DBS) of the subthalamic nucleus (STN) may have a beneficial effect on camptocormia in iPD. STN-DBS is known to partially remove disturbances in proprioception (Maschke et al., 2005). Unfortunately, the literature report that about half of iPD patients with camptocormia who underwent STN-DBS showed no improvement in bent back angle (Chieng et al., 2015; Srivanitchapoom and Hallett, 2016). A recent retrospective study investigated whether DBS of the subthalamic nucleus would in principle be able to relieve bent back in camptocormia of iPD patients and which factors were correlated with the outcome (Schulz-Schaeffer et al., 2015). Twenty-five iPD patients suffering from camptocormia who underwent DBS of the STN were given a standardized questionnaire. Information from medical records and family members were used additionally. Thirteen patients were classified as responders who showed improvement in the bending angle of the spine after STN-DBS of at least 50%; 12 were classified as non-responders. Responders and non-responders did not differ statistically with regard to the male-to-female ratio, age at PD onset, period of PD before camptocormia, bending angle before DBS, UPDRS III before DBS or levodopa-equivalent dose before DBS. The positive predictive factor related to an improvement in the angle of bent back using STN-DBS was a short duration of camptocormia symptoms. All patients with a captocormia duration of up to 20 months improved, whereas all but one with camptocormia duration of over 40 months did not. A scar-like mechanism of fibrosis and fatty degeneration in long-term diseased paraspinal muscles may hinder the effect of DBS on these muscles.

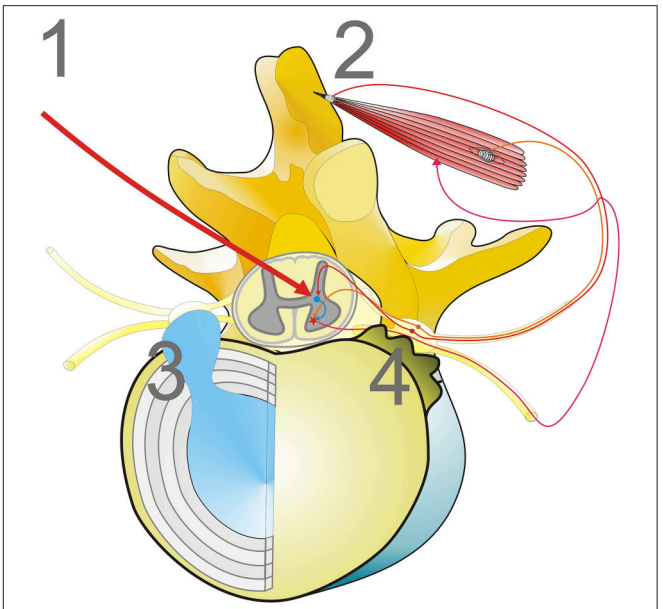

FIGURE 1 | Mechanisms disturbing the proprioceptive polysynaptic reflex arch can cause characteristic myopathological lesions. (1) Central dysregulation, frequently associated with basal ganglia diseases, i.e., iPD; (2) Loss of muscle tension input to the polysynaptic reflex arch, while the reflex arch is functionally intact (i.e., tenotomy; Popeye syndrome); (3, 4) Mechanical irritation of dorsal root ganglia or dorsal root nerves, for example by disc herniation (3) or spondylophytes of disc plates (4) that occur as a phenomenon of aging.

CONCLUSION

The polysynaptic reflex arch of the spinal cord integrates sensory information to motoric output. The sensory input comes from Golgi tendon organs, muscle spindles and joint receptors. Their information is not only integrated to motoric output, but may raise awareness such that motoric output can be influenced voluntarily. The sense of joint position, of movement (kinesthesia) and the sense of muscle strength are part of our self-awareness or proprioception. It is known that proprioception is impaired in some diseases of the central nervous system, for example in Parkinson's disease. Disturbances in proprioception—regardless of whether they originate centrally through neurodegenerative diseases or peripherally due to mainly age-related alterations—may cause characteristic myopathological changes in the axial musculature responsible for maintaining an upright body position, in extreme cases resulting in the syndrome known as camptocormia. Thus disturbances in the proprioceptive polysynaptic reflex arch may reflect mechanisms of neurodegeneration or mechanisms of aging.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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REVIEW
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Role of VEGF, Nitric Oxide, and Sympathetic Neurotransmitters in the Pathogenesis of Tendinopathy: A Review of the Current Evidences

*Sebastiano Vasta, Alberto Di Martino *, Biagio Zampogna, Guglielmo Torre, Rocco Papalia and Vincenzo Denaro*

Department of Orthopaedic and Trauma Surgery, Campus Bio-Medico University of Rome, Rome, Italy

Chronic tendinopathy is a painful common condition affecting athletes as well as the general population undergoing to tendon overuse. Although its huge prevalence, little is known about tendinopathy pathogenesis, and even cloudier is its treatment. Traditionally, tendinopathy has been defined as a lack of tendon ability to overcome stressing stimuli with appropriate adaptive changes. Histologic studies have demonstrated the absence of inflammatory infiltrates, as a consequence conventional antinflammatory drugs have shown little or no effectiveness in treating tendinopathies. New strategies should be therefore identified to address chronic tendon disorders. Angiofibroblastic changes have been highlighted as the main feature of tendinopathy, and vascular endothelial growth factor (VEGF) has been demonstrated as one of the key molecules involved in vascular hyperplasia. More recently, attention has been focused on new peptides such as Substance P, nitric oxide, and calcitonin gene-related peptide (CGRP). Those new findings support the idea of a nerve-mediated disregulation of tendon metabolism. Each of those molecules could be a target for new treatment options. This study aimed to systematically review the current available clinical and basic science in order to summarize the latest evidences on the pathophysiology and its effect on treatment of chronic tendinopathy, and to spread suggestions for future research on its treatment.

Keywords: tendons, tendinopathy, VEGF, nitric oxide (NO), nociceptive substance P (SP), neurotransmitter agents

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Paolo Bonaldo,
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Alessandro Piperno,
University of Rome Tor Vergata, Italy

****Correspondence:***
Alberto Di Martino
dimartino.cbm@gmail.com

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INTRODUCTION

With the increasing number of amateur sport practitioners, a growing prevalence of tendinopathy has been recorded in the last few years in Europe and United States (Andia and Maffulli, 2015), affecting mainly Achilles tendon, rotator cuff, extensor tendons at the lateral epicondyle of the elbow and patellar tendon (Huang et al., 2004; Papalia et al., 2013). It has been described as an altered healing response of the tendon to stressful conditions (Papalia et al., 2013), including repetitive microtrauma, overloads, and acute and chronic injuries. The huge prevalence, the invalidating symptoms, the long time needed to return to activities, and the challenging management of chronic tendinopathies, rise concerns about the best treatment for these diseases. Apart from surgery, a prompt clinical benefit is usually achieved by bed rest, topic, or systemic drugs (including, NSAID's) (Crisp et al., 2008; Zhang et al., 2013), taping, cryotherapy, or modalities such as laser therapy and shockwaves (Steunebrink et al., 2013). However, an integrated treatment

that considers the biologic pattern related to tendinopathy is far to be defined (Andia and Maffulli, 2015). At present, a growing effort in the scientific literature is aimed to understand the whole array of molecular and structural changes that are involved in the pathogenesis of tendinopathy. Despite the recent development and diffusion of biological therapies such as Platelet Rich Plasma (PRP) and mesenchymal stem cells, most surgeons ignore the basic science that underlies the molecular targets of these treatments.

At the end of the biological processes that lead to tendon tissue healing, we acknowledge the synthesis of collagen, which is achieved by an increase in the number and in the function of fibroblasts. This process has already been investigated in several studies (Connell et al., 2009; Clarke et al., 2011; Ahmad et al., 2012) reporting on the results of direct injection of dermal fibroblasts (Ahmad et al., 2012) or mesenchymal bone marrow cells (Ellera Gomes et al., 2012). Next to that, several other biologic items have been investigated, including the pathways of major molecules that have been found relevant in the pathogenesis of tendinopathy, either considering the role of vascular supply and innervation of the tendon. Specifically, concerning the vascular function, the molecules that have been investigated include the Vascular Endothelial Growth Factor (VEGF), the Hypoxia Inducible Factor (HIF), and the Nitric Oxide (NO); regarding the neurotransmitters, scientists mainly studied substance P (SP), Neurokinin-1 (N-1) and Calcitonin Gene-Related Peptide (CGRP). Several experimental studies aimed to stimulate the healing pathways acting directly on these molecular targets, showing promising results.

The aim of the present review of the literature is to explore the current literature regarding the role of vascular and neuronal molecular pathways in the pathogenesis and healing process of tendinopathy. Furthermore, the therapeutic implications of those pathways have been evaluated and shown in the below paragraphs.

MATERIALS AND METHODS

Articles research has been carried out using PubMed online database (http://www.ncbi.nlm.nih.gov/pubmed), between June and August 2015. The combinations of key-words used were the following: "("tendinopathy"[MeSH Terms] OR "tendinopathy"[All Fields]) AND ("vascular endothelial growth factor a"[MeSH Terms] OR "vascular endothelial growth factor a"[All Fields] OR "vegf"[All Fields])," "("tendinopathy"[MeSH Terms] OR "tendinopathy"[All Fields]) AND ("substance p"[MeSH Terms] OR "substance p"[All Fields] OR "p substance"[All Fields])," "("tendinopathy"[MeSH Terms] OR "tendinopathy"[All Fields]) AND ("neurotransmitter agents"[Pharmacological Action] OR "neurotransmitter agents"[MeSH Terms] OR ("neurotransmitter"[All Fields] AND "agents"[All Fields]) OR "neurotransmitter agents"[All Fields] OR "neurotransmitter"[All Fields])." The search was aimed to retrieve any level of evidence studies concerning molecular pathways involved in pathogenesis of tendinopathy, clinical associated features and therapeutic implications. Both clinical and experimental *in vivo* and *in vitro* studies were included. No study types were excluded except for literature reviews and case reports. No time interval for publication was set. Of each of the retrieved articles, the whole bibliography was carefully checked to enrich the research with possible studies relevant for the present work. Results of the studies were read, analyzed, and tabulated. The included studies have been divided into three categories: vascular function, nervous function, and therapeutic studies. The study selection process was carried out as shown in **Figure 1**.

RESULTS

Vascular Function (Table 1)

Behavior of Endogenous Angiogenetic Factors in Tendinopathy

Studies have demonstrated that neovascularization is one of the main features of tendinopathy (Zanetti et al., 2003; Rees et al., 2006) and that it is mainly a VEGF-driven process (Abraham et al., 2002; Yamazaki and Morita, 2006). In normal, asymptomatic adult tendons, the expression of VEGF is mostly suppressed, while in chronic overused tendons, VEGF expression is markedly increased in the early and late phases of the overuse process (Perry et al., 2005). Many *in vitro* studies have showed a close relation between cyclic strain and increased VEGF expression. In a recent study (Mousavizadeh et al., 2014), it has been found that cyclic strain applied to *in vitro* tendon cells yields to angiogenetic factors gene expression and synthesis, including angiopoietin like-4 (ANGPTL4), fibroblast growth factor-2 (FGF-2), cyclooxygenase-2 (COX-2), sphingosine kinase-1 (SPHK1), (transforming growth factor) TGF-α, VEGF-A, and VEGF-C. Comparable results were obtained by Petersen et al. (2004), that showed an increased synthesis of VEGF and Hypoxia Induced Factor-1α (HIF-1α) in fibroblasts cultures of rat tendons when an intermittent strain stress was applied. Nakama et al. (2006) found increased levels of VEGF and VEGFR-1 in tendons stimulated with continuous loading, compared to unstimulated tendons ($p = 0.0001$ and $p = 0.046$ respectively). An *in vivo* study on rabbits, by means of specific exercise protocols, Andersson et al. (2011b) showed an increase in tenocyte number and vascularization at 3 and 6 weeks. At the 6-week control, also the VEGF mRNA resulted overexpressed. Another *in vivo* study, by Sahin et al. (2012), investigated the expression of VEGF, HIF-1α, and MMP-3, and analyzed the tendons' biomechanical features in a tendinopathy model realized by freezing the rat's patellar tendon. At 7 days from the intervention, all the above-mentioned factors were increased and the angiogenesis was abundant. Furthermore, the biomechanical analysis showed a significant reduction in maximum stress and Young's module of the frozen tendons when compared with normal tendons. In human subjects, Scott et al. (2008), found that VEGF was overexpressed in patellar tendinopathy and not in normal tendons; moreover higher VEGF levels were detected in patients with symptoms of less duration (12 vs. 32.8 months). Chen et al. (2008) found postoperative increased levels of connective tissue growth factor (CTGF), TGF-β, VEGF, and Insulin-like growth factor-1 (IGF-1) after tendon repair in chicken models. Conversely, b-FGF

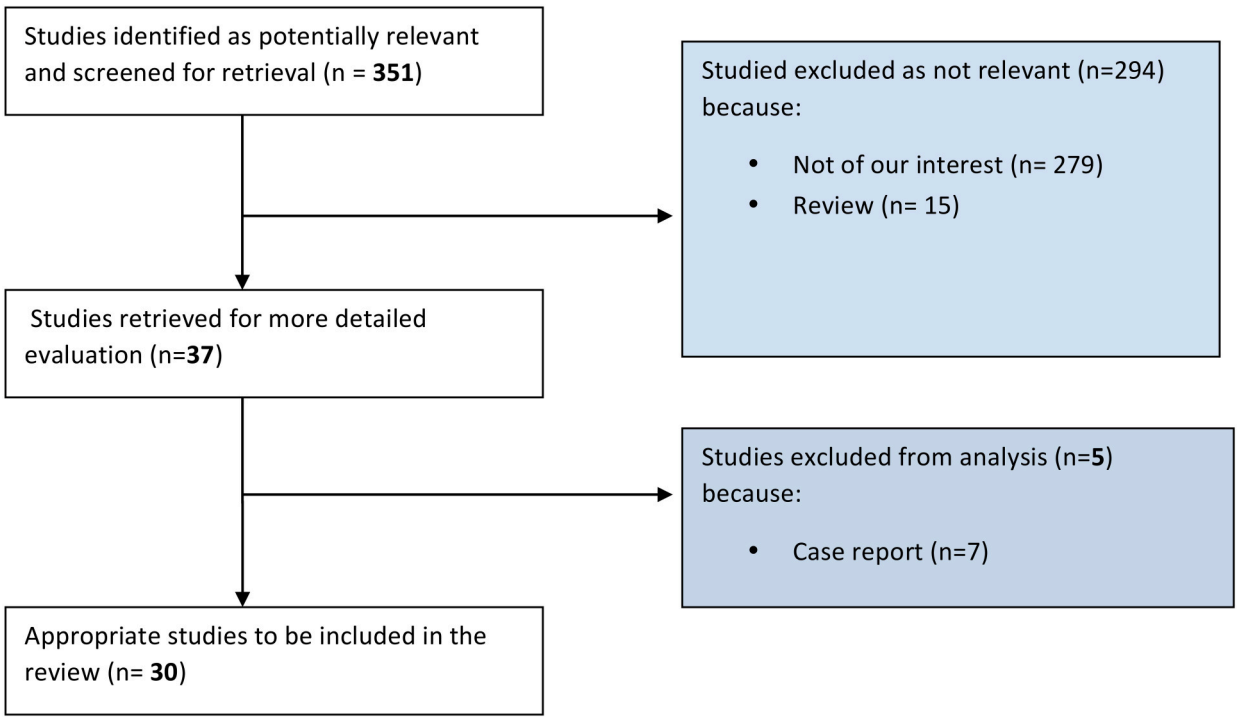

FIGURE 1 | Selection process of the studies.

expression was downregulated, and Platelet-Derived Growth Factor (PDGF) was slightly elevated. Consistently, Petersen et al. (2004) found an increase in VEGF levels after Achilles' tendon tenotomy in sheeps. In this model, the splice variants VEGF120 and VEGF164 were especially increased, at 3 and 24 weeks respectively. These findings demonstrate that VEGF is involved in the in mechanism of angiogenesis and Achilles' tendon repair.

Behavior of Endogenous Nitric Oxide in Tendinopathy

Studies concerning nitric oxide (NO) and nitric oxide synthase (NOS) expression have been carried out, in order to evaluate endothelial activation during tendinopathy and its effects on tendon tissue. An overuse protocol applied to supraspinatus tendons was evaluated by Szomor et al. (2001), that found an increased expression of both inducible NOS (iNOS) and constitutive NOS (eNOS), compared to controls. The same authors found increased expression of iNOS and eNOS in human rotator cuff specimens harvested during rotator cuff repair surgeries (Szomor et al., 2001). However, NO seems to be important in new tissue synthesis during tendon healing. The NO-paracetamol association has been added to tendon tissue during *in vitro* Achilles tendon healing harvested from a murine model. Increased amount of collagen and improved collagen reorganization were found (Murrell et al., 2008). Murrell et al. (1997) showed a five-fold increase in NO synthase activity 7 days after surgical division of rat Achilles tendons, with levels approximating the baseline at day 14. Moreover, when the activity of NO synthase was inhibited, a significant reduction in cross-sectional area and in the load to failure of the tendons was observed. Lin et al. (2001) found similar outcomes after Achilles tendon surgery in rats, showing increased expression of all the isoforms of NOS. A study by Xia et al. (2006b) evaluated the role of iNOS in the healing process of tendinopathy from a murine model. Three groups were compared: group 1: wild-type ($iNOS^{+/+}$); group 2: knock-out ($iNOS^{-/-}$); and group 3: knockout ($iNOS^{-/-}$) + systemic NO synthase inhibition through aminoguanidine (AG) administration. When systematically inhibiting the NO synthase in $iNOS^{-/-}$ mice (group 3), the cross-sectional area of the healing Achilles tendon was significantly reduced. However, no significant differences were found between the wild-type (group 1) and the knock-out mice (group 2) concerning both the cross-sectional area and biomechanical features of the healing Achilles tendon. Moreover, the same authors (Xia et al., 2006a) demonstrated an increased collagen synthesis in cultures of human rotator cuff tenocytes when cells were exposed to exogenous NO (in the form of S-nitro-N-acetyl-penicillamine), and when these were transfected with the iNOS gene via an adenovirus vector.

Nervous Function and Neuronal Stimulation (Table 2)

Behavior of Endogenous Neurotransmitters in Tendinopathy

It has been recently considered a role for neurotransmitters in the evolution of tendinopathy. The main molecule that has been investigated is Substance P (SP), which is known to play

TABLE 1 | Vascular function studies.

Study	Year	Investigated molecules	Type of study	Type of sperimentation	Role in pathogenesis	Role in healing
Anitua et al.	2005	IGF-1, TGF-β-1, PDGF-AB, VEGF, HGF, EGF	*In vivo*	Comparison of the effect of platelet-poor and platelet-rich cloth releasates (PPCR, PRCR) and platelet-poor plasma (PPP) on tendon cell cultures		PRCR induces tenocytes proliferation more than PPCR
Mousavizadeh et al.	2014	ANGPTL4, FGF-2, COX-2, SPHK1, TGF-α, VEGF-A, and VEGF-C	*In vitro*	Strain protocol applied to tendon cell cultures. Measurement of gene expression and proteins synthesis	Tendon cell strain induces expression of angiogenetic growth factors	
Petersen et al.	2003	VEGF, HIF-1α	*In vitro*	Strain protocol applied to cell culture (3T3 fibroblasts and rat Achilles tendon cells) and measurement of factors expression	Cell strain increased VEGF and HIF-1α concentrations in culture supernatant	
Asundi et al.	2007	MMP-1, MMP-3, VEGF, CTGF, COX-2, IL-1β, COL-III, FBRN	*In vivo*	*In vivo* cumulative load of tendon vs. control and mRNA expression measurement	No difference of mRNA expression in loaded and unloaded tendons	
Sahin et al.	2012	VEGF, HIF-1α, MMP-3	*In vivo*	*In vivo* frozen model of tendinopathy was evaluated with immunohistochemistry		HIF-1α, VEGF, and MMP-3 increased after intervention, and then decreased after 14 and 28 days
Liang et al.	2012	HIF-1α, VEGF, Bnip3, Bcl-2, Bcl-xL	*In vitro*	Hypoxia protocol on tenocytes cultures. Measurement of HIF-1α, VEGF mRNA and pro- and anti-apoptotic peptides	Hypoxia induced overexpression of HIF-1α, VEGF, Bnip3, but not of Bcl-2 and Bcl-xL	
Hao Chen et al.	2008	CTGF, TGF-β, VEGF, IGF-1, bFGF, PDGF-B	*In vivo*	Surgical repair and post-operative harvesting of tendon on *in vivo* chicken model		CTGF TGF-β, VEGF. and IGF-1 were highly expressed during early healing period, bFGF was downregulated, and PDGF-B was minimally expressed
Andersson et al.	2010	VEGF mRNA	*In vivo*	Electrical *in vivo* stimulation and passive flexion of triceps surae on rabbit model. Tenocytes number, VEGF mRNA, and vascular density evaluation	Increased tenocyte number at 3 and 6 weeks of exercise. Increased vascularization in rabbits with 3-weeks exercise. VEGF mRNA increased at 6 weeks	
Petersen et al.	2003	VEGF, VEGFR-1, and VEGFR-2	*In vivo*	Tenotomy of Achilles tendon in *in vivo* sheep model. VEGF levels evaluation during healing		VEGF resulted increased in tenotomised tendons and not in healthy ones. Splice variants VEGF120 and VEGF164 were increased respectively at 3 and 24 weeks

(Continued)

TABLE 1 | Continued

Study	Year	Investigated molecules	Type of study	Type of sperimentation	Role in pathogenesis	Role in healing
Scott et al.	2008	VEGF	*In vivo*	Human patellar tendons with and without tendinosis were harvested. VEGF levels were assessed	VEGF was overexpressed in intimal cells of the vessels in affected tendon	
Nakama et al.	2006	VEGF, VEGFR-1, CTGF	*In vivo*	Cyclical loading applied to flexor digitorum profundus in rabbits. Expression of VEGF and receptor, CTGF were evaluated in loaded tendons and contralateral ones	VEGF, VEGFR-1, and CTGF were higher in loaded tendon, than in controls	
Sozomor et al.	2006	iNOS, eNOS	*In vivo*	Overuse protocol applied to supraspinatus tendon in animal model vs. controls, isoforms of the NOS measurement	eNOS and iNOS were four-fold increased when compared to controls	
Xia et al.	2006	iNOS, NO	*In vivo*	Achilles tendon repair in murine model $iNOS^{-/-}$ vs. $iNOS^{-/-}$ with aminoguanidine (iNOS inhibitor) vs. wildtype. Microscopic healing process evaluation and NO levels measurement		Healing process was comparable between $iNOS^{-/-}$ and WT at 7 days from surgery. AG delayed healing in $iNOS^{-/-}$, but NO levels were higher in WT group
Xia et al.	2006	NO, iNOS	*In vitro*	Tendon cells from rotator cuff were cultured and stimulated with NO and iNOS gene carrier. Enzimatic inhibition was performed		Low NO doses promoted fibroblasts proliferation, high NO doses inhibited proliferation
Lin et al.	2001	nNOS, iNOS, eNOS	*In vivo*	Achilles tendon surgery in rats and mRNA level of NOS isoforms measurement		All NOS isoforms were increased during healing

several roles in proliferation of fibroblasts, angiogenesis, and pain transmission (Andersson et al., 2011a). Other molecules that have been investigated include Acetylcholine (Ach) and its receptors, Neuropeptide Y (NPY) and its receptors, and Glutamate (Glu) and its receptors. A recent study by Fong et al. (2013) evaluated the effect of Ach on Achilles tendon cell cultures, showing an increased number of viable cells and proliferation, and a phosphorylation of the epidermal growth factor receptor (EGFR) and the extracellular signal-regulated kinases ERK1/2 that are responsible of the Ach effects. NPY and receptors Y1 and Y2 were investigated by Bjur et al. (2009), that demonstrated by immunohistochemistry an increased expression of Y1 and high levels of NPY in human Achilles tendinopathy cultures. The same Authors (Bjur et al., 2008) showed that tenocytes from tendinopathic Achilles displayed Tyrosine Hydroxylase (TH) positive immunoreactions and reactions for TH mRNA, in addition to α1-adrenoreceptors, showing therefore evidence of local catecholamine production, not only at the protein level but also at the mRNA level. The authors advanced the hypothesis that tenocytes produce catecholamines with a possible autocrine/paracrine effects and that they can respond to sympathetic transmitters. Adrenergic stimuli can have an influence on degenerative/apoptotic events but can also induce cell proliferation (Zhang and Faber, 2001). These findings are supported by the study of Danielson et al. (2007) that showed in addition to immunoreactions for TH and α1-adrenoreceptors, a positivity for α 2A-, and β1-adrenoreceptors and for neuropeptide Y. High levels of these receptors were also detected in the blood vessels' walls. The same Authors (Danielson et al., 2008) in a further study on biopsies from paratendinous area dorsal to the proximal patellar tendon of tendinopathic patients who underwent arthroscopy, confirmed the presence of the immunoreaction patterns and specifically demonstrated a marked immunoreaction for sympathetic markers in the small and large blood vessels surrounding the abnormal tendon tissue. Schubert et al. (2005) found in human Achilles tendinopathy samples nerve fibers positive to Nociceptive substance P (SP), and these were significantly increased together with an inflammatory infiltration of B and T lymphocytes. Backman et al. in two studies (Backman and Danielson, 2013; Backman et al., 2014)

TABLE 2 | Nervous function and neuronal stimulation studies.

Study	Year	Investigated molecules	Type of study	Type of sperimentation	Role in pathogenesis	Role in healing
Fong et al.	2012	Ach, mAch-R, ChAT, VAchT	*in vitro*	Achilles tendon cells cultures treated with Ach. Immunohistochemical evaluation of cells immunopatterns and EGFR activation		Ach adimistration increase viable cells number, proliferation and activation of the EGFR and ERK1/2 pathways
Bjur et al.	2009	NPY and receptors Y1 and Y2	*in vitro*	Achilles tendon cell culture from human tendon with tendinosis. Immunohistochemical evaluation	Increased expression of Y1 receptor and NPY was found	
Schubert et al.	2005	SP	*In vivo*	Achilles tendon tissue with tendinosis vs. spontaneous rupture without tendinosis were examined microscopically	Increased number of SP positive fibers was found in tendinosis tendons, and not in spontaneous rupture	
Backman et al.	2011	SP, NK-1R	*In vivo*	Overuse protocol applied *in vivo* to rabbit Achilles tendons for 1, 3 or 6 weeks vs. controls	Increased SP expression at each time. SP and NK-1R were found in blood vessel walls. NK-1R was also found on tenocytes	
Lui et al.	2010	SP, CGRP	*In vivo*	Collagenase-induced tendinopathy in patellar tendons in rats. Double stance duration before sacrifice assessment and immunohistochemical evaluation		Increased SP and CGRP were found at 2 week, but not at 4 weeks and 8 weeks. A second peak was at 12 weeks and 16 weeks. SP and CGRP changes were consistent with double stance duration
Schizas et al.	2012	SP, NMDAR1, p-NMDAR1, mGluR1, 5, 6, and 7	*In vivo*	Immunohistochemical evaluation of human patellar tendon tissue with exercise-related tendinopathy	Increased expression of SP, NMDAR1, p-NMDAR1 and mGlutR5 was found in comparison with controls. Co-localization of SP and NMDAR1 was found	

showed that SP reduces the Anti-Fas-induced apoptosis in healthy human tenocytes, and that this antiapoptotic effect of SP is mediated through NK-1 R and Akt-specific pathways. These findings support the role of SP in inducing the marked hypercellularity seen in tendinopathy. The same Authors found (Backman et al., 2011) in an animal model of Achilles tendon overuse, that already after 1 week of overloading, the SP levels were significantly elevated compared to a control group. Zhou et al. (2014) showed that the injection of exogenous SP in Achilles tendons of rats yielded to significantly increased proliferation of pluripotent tendon cells (PTCs). Moreover, the reverse transcription polymerase chain reaction (RT-PCR) showed that SP upregulated the expression of non-tenocyte genes but downregulated the expression of tenocyte-related genes. These findings indicate that SP is responsible for enhanced PTCs' proliferation, promotes non-tenocyte differentiation and plays an important role in the development of tendinopathy. In a study of Lui et al. (2010) after collagenase-induced tendinopathy of the patellar tendon, double stance duration was evaluated and then immunohistochemical evaluation was carried out. Increased SP and CGRP were found at 2 weeks, but not at 4 weeks and 8 weeks. Other peaks were found at 12 weeks and 16 weeks. The increased expression of SP and CGRP positively correlated with the duration of double stance. The presence of increased blood vessels and sympathetic nerve components in chronic patellar and Achilles tendinopathy was also confirmed by the studies of Andersson et al. (2007) and from Lian et al. (2006). Anderson and colleagues undertook a histological study of the ventral portion of tendinopathic Achilles tendons and demonstrated the presence of large and small arteries and nerve fascicles. The nerve fascicles contained sensory nerve fibers, positive for SP and CGRP staining, and sympathetic nerve fibers (Andersson et al., 2007). Lian and colleagues showed that chronic painful patellar tendons exhibited increased occurrence of sprouting nonvascular sensory, substance P–positive nerve fibers compared to a control group (Lian et al., 2006). In a study by Schizas et al. (2012), a relevant correlation was found between SP and glutamate receptors in human patellar tendons affected by

exercise-related tendinopathy. The expressions of N-methyl-D-aspartate receptor type 1 (NMDAR1) and its phosphorylated form (P-NMDAR) were evaluated together with SP levels and mGlu receptors family levels. They found an increased expression of SP, NMDAR1, p-NMDAR1, and mGlutR5, when compared to controls. Furthermore, a significant co-localization of SP and NMDAR1 was found exclusively in tendinopathic tendons and not in healthy controls, suggesting a possible role of SP in stimulating NMDAR1. These findings are supported by previous studies such as those from Alfredson et al. (2001a,b), which found both free glutamate and glutamate NMDAR1 receptors in human Achilles' tendons from patients with chronic Achilles tendinopathy, and free glutamate and glutamate NMDAR1 receptors, but not PGE_2, in patellar tendons with tendinopathy in respect to normal tendons.

Consistently with SP's potent effect on stimulating proliferation of fibroblasts and endothelial cells, Burssens et al. (2005) demonstrated as paratendinous injections of SP after operative repair of the Achilles tendon in rats, significantly enhanced tendon healing compared to controls. Similar results were achieved by other authors. Steyaert et al. (2010) reported that exogenous injections of SP yielded to enhanced angiogenesis and fibroblast proliferation, and boosted the endogenous substance P effects for fibroblast proliferation via an autocrine/paracrine stimulation, though SP didn't stimulate sensory nerve ingrowth. Bring et al. (2012), after depleting SP levels through Capsaicin in rats who underwent surgical transection of the Achilles tendon, showed that rats with higher residual SP levels developed improved tensile strength and stress at failure in the healing of Achilles' tendons. Carlsson et al. (2011) showed that substance P injections enhance tissue proliferation and regulate sensory nerve ingrowth on sutured rat's Achilles tendon, previously torn. The contrasting effects of Substance P could find a possible explanation thanks to the finding from a recent *in vitro* study by Zhou et al. (2014). These authors demonstrated that adding high-doses of SP (5.0 nmol) to patellar tendon tissue yielded to tendinopathic changes. Low doses of SP (0.5 nmol) boosted up the tenogenesis process compared with saline injection (control group) and the high-dose SP group. These findings suggest that SP has a possible dose-dependent effect: low doses could be advantageous for tendon healing, while high SP doses can be responsible for tendinopathic evolution.

Effects of Exogenous VEGF, NO, and SP on Tendinopathy (Table 3)

Almost all the previously described factors have been investigated in experimental or clinical setting to assess the biological response to this therapy on tendinopathy.

The administration of the splice VEGF-111 in artificially injured Achilles tendon was investigated by Kaux et al. (2014), who showed a significantly higher strength-to-rupture of the tendons at 15 and 30 days. Similar results were obtained by Zhang et al. (2003), which found higher tendon strength in rats treated with VEGF injection after Achilles tendon rupture and repair, compared to the controls.

The use of NO has been investigated in several experimental and clinical settings on different types of tendinopathies, and it has been administrated clinically through a transdermal route. Paoloni and colleagues demonstrated the beneficial effects of glyceril trinitrate (GTN) patches on elbow extensors tendon (McCallum et al., 2011) and on Achilles' tendon (Paoloni et al., 2004), with improvement of pain and function at follow-up, especially in reducing tenderness and increasing muscle's peak force and total work. Comparable results were obtained by Steunebrink et al. (2013), evaluating GTN application plus eccentric exercise for patellar tendinopathy. Double-blinded randomized trials (RCTs) have been conducted to assess whether NO is useful in tendinopathy. Specifically, Paoloni et al. (2003) investigated 86 patients with chronic extensor tendinopathy at the elbow, undergoing to rehabilitation plus either topical NO application in the form of GTN patch or placebo. Those patients from the treatment group showed significantly reduced pain at 2 weeks, as well as reduced pain during activity, reduced tenderness at the lateral epicondyle at 6 and 12 weeks and increased wrist extensor mean peak force at 24 weeks. In a further study (McCallum et al., 2011) a subset of patients from the original trial was follow-upped at 5 years, and patients from the treatment group did not score better than those from the placebo group. In a double-blinded RCT on patients affected by Achilles tendinopathy, two groups were compared, either undergoing to rehabilitation plus either GTN patch or placebo. The treatment group demonstrated significantly reduced Achilles activity pain at 12 weeks, reduced night pain at 12 weeks, reduced tenderness at 12 weeks, decreased pain scores with the hop test at 24 weeks, and an increase in ankle plantar flexor mean total work at 24 weeks (Paoloni and Murrell, 2007). The same patients were further assessed at 3 years, and the results showed as 88% of patients with GTN treatment were asymptomatic at 3 years compared with 67% of patients treated with tendon rehabilitation alone (Paoloni and Murrell, 2007).

The SP administration in tendinopathy has been extensively studied by Burssens et al. (2005) who studied the effects of NO in surgically repaired Achilles tendon of rats. In the group receiving exogenous SP, increased fibroblasts proliferation, angiogenesis and collagen fibers organization were found at 7 days post-operatively showing a faster healing of Achilles tendons, even though there were no differences at 14 days with the control group. An interesting study from Mousavizadeh et al. (2015) showed that exposing human tendon cells to dexamethasone resulted in a time-dependent reduction of mRNA for SP. However, it should be taken into account as there is huge evidence that steroids negatively affect viability, migration, proliferation, and collagen synthesis of both human and animal tenocytes (Tsai et al., 2003; Wong et al., 2003, 2009). Zhang et al. (2013) in fact recently demonstrated that dexamethasone exposure induced non-tenocyte differentiation of human tendon stem cells, determining a nearly complete suppression of collagen type I expression, and an up-regulation of non-tenocyte related genes (PPARg and Sox-9). Whether, this mechanism may be mediated by an under-regulation of SP levels, has not yet been demonstrated.

TABLE 3 | Therapeutic studies.

Study	Year	Investigated molecules	Type of study	Type of sperimentation	Role in healing
Lu et al.	2008	VEGF	*In vivo*	Partial patellectomy on *in vivo* rabbit model. Healing stimulation through LIPUS vs. controls. VEGF expression measurement	VEGF expression resulted more increased in LIPUS group than controls
Liang et al.	2012	HIF-1α, VEGF, Bnip3, Bcl-2, Bcl-xL	*in vitro*	Hypoxia protocol on tenocytes cultures. Measurement of HIF-1α, VEGF mRNA and pro- and anti-apoptotic peptides	Insulin or PRP showed protective effects on cell death
Kaux et al.	2014	VEGF111	*In vivo*	VEGF111 vs. saline injection in Achilles tendon after artificial lesion in murin model	Force for tendon rupture at 15 and 30 days was higher in VEGF111 group
Zhang et al.	2003	VEGF, TGF-β, PDGF, bFGF, IGF-1	*In vivo*	Transection and suture repair of Achilles tendon in murin model. VEGF injection at repair site vs. saline injection. TGF-β levels measurement	Tensile strength was higher in tendons treated with VEGF than controls in low terms. TGF-β was increased in VEGF group. PDGF, bFGF, IGF-1 were comparable
Paoloni et al.	2005	GTN	*In vivo*	Local application of GTN patch for elbow extensors tendinopathy vs. controls. Measurement of pain, tenderness, peak force and total work	Exogenous NO administration improve healing of the tendons
Paoloni et al.	2004	GTN	*In vivo*	Rehabilitation plus local application of GTN patch for Achilles tendinopathy vs. controls. Measurement of pain, tenderness, peak force, and total work	Exogenous NO administration improve healing of the tendons
Steunebrink et al.	2013	GTN	*In vivo*	Eccentric exercise plus GTN patch application on patellar tendon vs. controls (exercise only)	Exogenous NO administration improve healing of the tendons
Murrell et al.	2008	NO-paracetamol	*in vitro*	*in vitro* administration of NO-paracetamol association to tendon cell culture	Improved collagen production and reorganization
Burssens et al.	2005	SP	*In vivo*	SP (different doses) administration to rats after Achilles tendon repair vs. tiorphan and captopril vs. controls. Microscopic evaluation of fibroblasts proliferation, angiogenesis and collagen organization	In tendons treated with SP, fibroblasts proliferation was increased at 7 days, but no at further f.u. Similar evolution for angiogenesis and collagen orientation

DISCUSSION

The increasing knowledge concerning tendon dysfunction, clinically expressed as tendinopathy, leads to the individuation of a huge array of factors implied in the pathogenesis and repair mechanisms of this disease. Among single molecules and pathways involved in pathogenesis and healing process of tendinopathy, vascular, and neuronal factors play a major role (Papalia et al., 2013, 2015; Notarnicola et al., 2014). The first ones are mainly involved in angiogenesis and vascular activation, while the second ones concern signaling for cell proliferation, collagen organization and pain transmission (Alfredson et al., 2003). All these features are considered the core of the pathogenesis, although their proper timing through each step of the tendinopathy process still remains unclear. Some attempts have been performed to find out the exact time and way of activation of each specific pathway, quintessential to find a targeted therapy for the tendinopathy process. From a clinical point of view, tendinopathy represents an invalidating disease, with a severe impact not only on sport practice, but also on daily activities. The main challenge when facing with tendinopathy is to provide patients with a therapy that is effective and fast-acting and provides for durable outcome.

Some studies have investigated the role that VEGF has in both the pathogenesis and to the healing response of tendinopathy, also using VEGF and its splice variants as an efficient treatment (Zhang et al., 2003; Kaux et al., 2014), that resulted in fibroblast proliferation and angiogenesis stimulation (Kaux et al., 2014). It has been found that VEGF and its receptor are the earlier molecules expressed in tendinopathy (Nakama et al., 2006), and even though the vascular hyperplasia induced by VEGF may be considered a key factor in the pathogenesis of tendinopathy (Scott et al., 2008), it is also the main mechanism of healing to restore tissue integrity.

SP administration in surgically repaired rat tendons (Burssens et al., 2005), have also some advantages, especially in terms of quickness of action. In addition, SP should be considered the vault key that put together the vascular and neural function, since it is known that SP promotes vascular activation and vasodilatation, and also tissue hyperplasia (Zhou et al., 2014).

The local vascular activation is actually considered a still unclear chapter of the wide topic of tendinopathy. Studies have demonstrated an increased expression of NOS isoforms produced by endothelium in diseased tendons (Szomor et al., 2001). Conversely knock-out mice for iNOS did not show a severely impaired tendon healing response (Xia et al., 2006a). Moreover, it has been shown as NO has variable effects on tendon tissue depending on its concentration (Xia et al., 2006b). At present, transdermal administration of NO related drugs (namely GTN patches) have shown a positive short-term effect in tendon healing even in the clinical setting (Paoloni et al., 2003, 2004; Tsai et al., 2003; Mousavizadeh et al., 2015).

However, since VEGF and its pathways are the broadly known factors among a so complex pathogenesis, it should probably be the main factor to pay efforts on. An interesting research line is the regulation of VEGF action during the pathogenesis of tendinopathy (Lu et al., 2008; Andersson et al., 2011b; Kaux et al., 2014), which is assessing whether hypervascularization is truly beneficial for tendon healing, and if so, in what phases of the process. The possible answer to such contrasting data about each of the investigated molecule (VEGF, NO, SP,) could be the timing. Understanding the exact time of intervention of those molecules during the tendinophaty pathogenesis and the healing process could lead to a targeted and timed therapy to enhance healing.

CONCLUSIONS

Vascularization and neuronal transmission play a key role in determining the pathogenesis of the tendinopathy. The mainly known factors implied in the process are VEGF, Substance P, and Nitric Oxide, although their exact role in the mechanism of tendinopathy is not well determined. More research should be carried out, especially studies involving human subjects, in order to assess the timing of action of those factors, to find out how therapies targeted to the phase of the disease process may fasten the healing process and the clinical recovery.

AUTHOR CONTRIBUTIONS

AD, RP, and VD supervised the articles selection process and reviewed the final manuscript. SV, BZ and GT provided articles selection, manuscript writing, and table filling up.

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HYPOTHESIS AND THEORY
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Semaphorin 3A: A Potential Target for Low Back Pain

*Pengbin Yin †, Houchen Lv †, Lihai Zhang, Licheng Zhang * and Peifu Tang **

Department of Orthopedics, Chinese PLA General Hospital, Beijing, China

Low back pain is a common disorder. Pathological innervation and intervertebral disc degeneration are two major factors associated with this disease. Semaphorin 3A, originally known for its potent inhibiting effect on axonal outgrowth, is recently found to correlate with disease activity and histological features in some skeletal disorders. Based on its effects on innervation and vascularization, as well as enzyme secretion, we presume that semaphorin 3A may act as a potential target for low back pain.

Keywords: low back pain, semaphorin 3A, neuropilins, pathological innervation, inter-vertebral disc degeneration

INTRODUCTION

Low back pain (LBP) is a common disorder. Around 60% of the adult population suffers from back discomfort at some point in their lifetime (Hoy et al., 2012; Campbell and Colvin, 2013). However, a definite pathological cause to this sickness is still unknown. Prior research has reported various potential factors, which may be associated with back pain, such as mechanical changes, low pH throughout the disc, disc degeneration, cytokines etc. Anatomically, it has been proposed that degenerative joint disease and intervertebral disc degeneration are the most common factors. In addition, pathological innervation into the degenerative intervertebral disc is said to be closely related with pain of intervertebral disc origin (Luoma et al., 2000; Cheung et al., 2009; García-Cosamalón et al., 2010; Miyagi et al., 2014). Thus, most therapeutic research on low back pain focuses on inhibiting neural invasion and delaying disc degradation (Mantyh, 2014; Vasiliadis et al., 2014; Sakai and Grad, 2015).

Semaphorin 3A, originally known for its potent inhibition of axonal outgrowth, has been found to play pivotal roles in several other systems (Luo et al., 1993; Barresi et al., 2009; Shim et al., 2013; van Gils et al., 2013). Notably, several recent studies have suggested that the expression of semaphorin 3A and its receptors (neuropilins) correlates with disease activity and histological features in some skeletal disorders (Hayashi et al., 2012; Negishi-Koga and Takayanagi, 2012; Fukuda et al., 2013; Takagawa et al., 2013). Previous research has demonstrated that semaphorin 3A is associated with innervation and vascularization, enzyme secretion, and cartilage development in skeletal tissues (Gomez et al., 2005; Okubo et al., 2011; Fukuda et al., 2013). These effects contribute to physiological and pathological alteration of the skeletal system. Further research also indicates the important role of enzyme secretion in disc degradation (Canbay et al., 2013; Xu et al., 2014). Since semaphorin 3A has been proven to be involved in both processes, we question that if there is any correlation between semaphorin 3A and low back pain.

HYPOTHESIS

Based on the effects of semaphorin 3A on innervation and vascularization, as well as on enzyme secretion in the skeletal system, coupled with the understanding of pathophysiology of low back

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Edited by:
Cesare Faldini,
Istituto Ortopedico Rizzoli, Italy

Reviewed by:
Vijay Karkal Hegde,
Texas Tech University, USA
Ilaria Sanzarello,
Department of Orthopaedic and Traumatology, Italy

****Correspondence:***
Licheng Zhang
zhanglcheng218@126.com;
Peifu Tang
pftang301@126.com

† These authors have contributed equally to this work.

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pain, we hypothesize that semaphorin 3A may be a potential therapeutic target for low back pain.

EVALUATION OF THE HYPOTHESIS

It has been observed in both animal and human studies that with the progression of degeneration, sensory nerve fibers—which require a low level of chemical and mechanical stimuli to trigger pain—begin to innervate normally anural and avascular areas (Freemont et al., 1997; Miyagi et al., 2014). Research by Mantyh et al. showed that the administration of anti-nerve growth factors dramatically block the sprouting of these fibers, therefore significantly inhibited pain in mice (Mantyh et al., 2010). Therefore, an approach to inhibit pathological neural and vascular innervation in degenerative vertebral discs represents a new potential for pain management and treatment.

The mechanisms underlying the degeneration of intervertebral discs along with aging are complex, though MMP has been shown to play an important role. Research showed that the expression of MMPs is positively related to the severity of degeneration (Rutges et al., 2008; Xu et al., 2014). Based on their catabolic biological activities, the function of these enzymes is to maintain the integrity of the matrix by cooperating with other factors that perform anabolic activities (Le Maitre et al., 2007). However, in a pathological condition the balance disrupts and can lead to excessive degradation of disc components (Vo et al., 2013).

Semaphorin 3A, a prototypical class 3 secreted semaphorin, is a potent inhibitor of axonal outgrowth in a specific subset of sensory and sympathetic neurons and induces collapse of their growth cones. Research by Sotonye et al. showed that semaphorin 3A is highly expressed by healthy disc cells and decreased significantly in degenerate samples (Tolofari et al., 2010). Considering its inhibition of axonal outgrowth, semaphorin 3A may act as a biological barrier against neuronal ingrowth within healthy intervertebral disc. In addition, mRNA for semaphorin 3A receptors (neuropilins) was identified in healthy and degenerate tissues (Tolofari et al., 2010). Neuropilins have also been confirmed to bind to vascular endothelial growth factor (VEGF), which is a key regulator of normal and pathologic angiogenesis (Dai and Rabie, 2007). As VEGF and class 3 semaphorins compete for binding to neuropilins, reduction of semaphorin 3A may lead to increased binding of VEGF. Furthermore, binding of neuropilins to VEGF has been shown to result in promotion of the migration, proliferation, and tube formation of endothelial cells (Bates et al., 2003; Dai and Rabie, 2007; Roskoski, 2007; Staton et al., 2007). This process provides chemoattractive cues for vascular innervation, which may be associated with vascularization in degenerated intervertebral disc. All in all, semaphorin 3A is a potent inhibitor of both pathological innervation and vascular proliferation.

Research on lung cancer has further shown a negative correlation between protein expression levels of semaphorin 3A and MMP-14 (Zhou et al., 2014). In neurons, semaphorin 3A was also shown to consistently reduce MMP-3 expression and activity (Gonthier et al., 2009). Recent research on osteoarthritic cartilage has also shown that semaphorin 3A inhibited VEGF165-induced overexpression of MMPs (Okubo et al., 2011). These findings demonstrate the potential role of semaphorin 3A in negatively regulating MMP secretion. Although there is no direct evidence showing a relationship between the expression of MMPs and semaphorin 3A in patients with low back pain, the observations in other tissues provide some clues on the effects of semaphorin 3A on degenerative intervertebral discs.

Therefore, based on the potent effects of semaphorin 3A on repelling nerve ingrowth and vascular proliferation, as well as its negative regulation of MMPs, we hypothesize that semaphorin 3A may be a potential therapeutic target for low back pain.

AUTHOR CONTRIBUTIONS

All of the authors meet all 4 of the requirements as stipulated in the Guide for Authors. Substantial contribution to the concept and design of this study: Pengbin Yin, Peifu Tang, and Licheng Zhang; literature retrieval: Licheng Zhang, Houchen Lv; and manuscript drafting: Pengbin Yin.

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MINI REVIEW
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Mechanical Stimulation (Pulsed Electromagnetic Fields "PEMF" and Extracorporeal Shock Wave Therapy "ESWT") and Tendon Regeneration: A Possible Alternative

*Federica Rosso *, Davide E. Bonasia, Antonio Marmotti, Umberto Cottino and Roberto Rossi*

Department of Orthopaedics and Traumatology, AO Mauriziano Umberto I, Torino, Italy

The pathogenesis of tendon degeneration and tendinopathy is still partially unclear. However, an active role of metalloproteinases (MMP), growth factors, such as vascular endothelial growth factor (VEGF) and a crucial role of inflammatory elements and cytokines was demonstrated. Mechanical stimulation may play a role in regulation of inflammation. *In vitro* studies demonstrated that both pulsed electromagnetic fields (PEMF) and extracorporeal shock wave therapy (ESWT) increased the expression of pro-inflammatory cytokine such as interleukin (IL-6 and IL-10). Moreover, ESWT increases the expression of growth factors, such as transforming growth factor β(TGF-β), (VEGF), and insulin-like growth factor 1 (IGF1), as well as the synthesis of collagen I fibers. These pre-clinical results, in association with several clinical studies, suggest a potential effectiveness of ESWT for tendinopathy treatment. Recently PEMF gained popularity as adjuvant for fracture healing and bone regeneration. Similarly to ESWT, the mechanical stimulation obtained using PEMFs may play a role for treatment of tendinopathy and for tendon regeneration, increasing *in vitro* TGF-β production, as well as scleraxis and collagen I gene expression. In this manuscript the rational of mechanical stimulations and the clinical studies on the efficacy of extracorporeal shock wave (ESW) and PEMF will be discussed. However, no clear evidence of a clinical value of ESW and PEMF has been found in literature with regards to the treatment of tendinopathy in human, so further clinical trials are needed to confirm the promising hypotheses concerning the effectiveness of ESWT and PEMF mechanical stimulation.

Keywords: tendon, tendinopathy, mechanical stimulation, extracorporeal shockwaves therapy, pulsed electromagnetic fields, tendon regeneration

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Edited by:
Cesare Faldini,
Istituto Ortopedico Rizzoli, Italy

Reviewed by:
Archana Mukhopadhyay,
University of Kansas, USA
Francesco Traina,
Istituto Ortopedico Rizzoli, Italy

****Correspondence:***
Federica Rosso
federica.rosso@yahoo.it

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INTRODUCTION

Tendon disorders include both acute and chronic diseases, such as tendinopathy. It is know that the tendon tissue is poorly cellularized, with 5% of the normal tissue occupied by tenocytes that produce the extracellular matrix (ECM), based on type I collagen. Furthermore, along with tenocytes, the human tendons are also composed by tendon stem/progenitor cells (TSPCs), that guarantee to the

tendon the ability to repair and regenerate and help in maintaining the homeostasis (Bi et al., 2007).

There is a debate on the role of inflammation in the production of degenerative changes in the tendon tissue (Abate et al., 2009; Cook and Purdam, 2009). However, recent new findings demonstrated the presence of inflammatory elements in pathologic tendons, as well as the activation of matrix metallo-proteinases, and the involvement of mediators such as substance P, vascular endothelial growth factor (VEGF), and cyclo-oxigenase type II (COX2; De Mattei et al., 2003). Tendon healing normally occurs in three different phases: the acute inflammatory phase, for up to 3–7 days, in which the neo-angiogenesis occurs, followed by the proliferative phase, for up to 21 days, when intrinsic cell proliferation of epitenon and endotenon tenocytes and extrinsic invasion of cells from the surrounding sheath and synovial occur, simultaneously with collagen, fibronectin, and elastin production, and the third remodeling phase, for up to 2 years (De Palma and Rapali, 2006; Abate et al., 2009). Different studies evaluated the molecular mechanisms that promote the healing process, such as metalloproteinases (MMPs) and MMPs with thrombospondin motifs (ADAMTs), in association with their tissue inhibitors (TIMPs; Sharma and Maffulli, 2005). Particularly MMP-9 and MMP-13 participate in collagen degradation only, while MMP-2, MMP-3, and MMP-14 are involved in both collagen degradation and remodeling (Riley et al., 2002). Indeed, different growth factors may be involved in neo-vascularization and stimulation of fibroblast and tenocytes, such as VEGF, TGF-β1, fibroblast growth factors (FGFs), and Scleraxis (Scx; Sharma and Maffulli, 2006). Furthermore, Nitric Oxide (NO) may also be involved in the healing process (Murrell, 2007).

In this scenario, different conservative treatments were proposed to treat tendon degeneration and tendinopathy. In this manuscript, we will analyze the rationale of mechanical stimulation by Extracorporeal shock wave (ESW) and Pulsed electromagnetic fields (PEMF) for tendon regeneration, and its possible role in the treatment of different tendinopathies.

EXTRACORPOREAL SHOCK WAVE THERAPY (ESWT) FOR TENDON'S PATHOLOGY

Shock Waves' Definition and Mechanism of Action

A shock wave is a special, non-linear type of pressure wave with a short rise time (around 10 μs) and a frequency ranging from 16 to 20 MHz (Ogden et al., 2001). These waves have a positive and negative (low-pressure) phase. In the first phase, ESW may hit an interface, with their reflection, or may gradually pass and become absorbed. The second phase causes cavitation at the tissue interfaces, with bubbles formation that subsequently implode, generating a second wave (Ogden et al., 2001). The propagating wave increase the tissue density and, as a consequence, transmit direct mechanical perturbations to the tissue with effects on cell membrane polarization, radical formation, cell proliferation, and growth factor production. Low energy ESW with a shock number ranging from 200 to 300 impulses seem most suited for enhancing cell proliferation and metabolism and, subsequently, for clinical applications.

There are two type of shockwave therapy: the focused shockwave therapy (FSWT) and the radial shockwave therapy (RSWT). *Focused shockwaves* are characterized by a pressure field that converges at a selected depth in the body tissues, where the maximal pressure is reached. FSWT can be generated using three methods: electro hydraulic (EH), electromagnetic (EM), and piezoelectric (PE). In all the cases the wave is generated in water, because of the acoustic impedance of water and biologic tissue are similar (van der Worp et al., 2013). The difference between the three methods of generation is the time at which the shockwave forms (Coleman and Saunders, 1989). RSWs are characterized by a diverging pressure field, which reach the maximal pressure at the source, and they are not generated in water (van der Worp et al., 2013). A recent *in vitro* study of Notarnicola et al. pointed out the negative effect of RWS on bone metabolism (Notarnicola et al., 2012), while some evidences in literature suggests a positive effect of RWS on the enthesis of plantar fascia and on the degenerated areas of Achilles tendon structure (van der Worp et al., 2013).

The supposed mechanism of action of ESW rely on conformational changes in membrane proteins, such as the integrins and, subsequently, on intracellular signal generation that modify gene expression and release of growth factors. Indeed, shock waves-induced repair phenomenon is observed in presence of increased level of IL-6, which is able to stimulate fibroblast production of collagen and ECM components (Waugh et al., 2015). A transient increase of IL-1b expression and a prolonged increase of IL-8 one was also described, consistent with a modulation of initial inflammatory phase. Following these observations, it is not surprising that ESW may stimulate matrix metalloproteinase (MMP) activity. Specifically, an increased expression of MMP-2 and the pro-MMP-9 was demonstrated. Considering the well-known low level of basal repair and MMP activity (i.e., MMP-9) in degenerative tendinopathy, these concepts support the statement that ESWT may also favor tendon repair by increasing pro-MMP forms availability, allowing for greater pathological tendon remodeling by ECM-degrading enzymes and, ultimately, favoring tendon tissue regeneration. Indeed, MMPs are also capable to activate latent TGF-β sequestered in the ECM. Nevertheless, a great inter-individual variability in the response of MMP to ESW was observed. This may represent a possible biological explanation for the variations in clinical success rates of ESWT in different tendinopathies and may be a clue for recognize and identify the population of responders and non-responders to ESWT.

Vetrano et al. demonstrated that ESWT up-regulates the expression of collagen (mainly type I) and stimulate cell proliferation in primary cultured human healthy tenocytes (Vetrano et al., 2011). However, the same group compared the results of ESWT (at the dose of 0.14 mJ/mm^2) in cultured healthy and pathologic tenocytes, demonstrating that Scx and collagen type I were significantly diminished in the pathologic

tenocytes cultures. These results indicate that the natural trigger for healing may be delayed by ESW treatment, in order to promote cellular repair (Leone et al., 2012). Pre-clinical studies showed that ESW are able to increase VEGF, VEGF receptor Flt-1, endothelial nitric oxide synthase (eNOS), and proliferating cell nuclear antigen (PCNA) expression, consistent with the initial neo-vascularization process. Improved blood supply and early vascularity is associated with the initial leukocyte infiltration and the subsequent metabolism of the fibers in the putative tendon pathological area by means of ECM-degrading enzymes (Bosch et al., 2009). This early phase is followed by an ESW-driven transitory increase in TGF-β1 expression, later followed by persistent IGF-I expression, that leads to a controlled inhibition of macrophages-induced ECM degradation and inflammation and an enhanced ECM and collagen type I synthesis (Visco et al., 2014). Tendon cells proliferation was also associated in this repair sequence, as well as endogenous lubricin production by fibroblasts and tenocytes following growth factors stimulation (i.e., TGF-β1; Zhang, 2011). The ultimate result of ESWT is a simulation of cell metabolism, which may induce healing process in injured areas of the tendons (Chen et al., 2004).

Recently, soft-focused ESWT was proposed, in order to deliver the energy in a larger area (Kuo et al., 2009). In the *in vitro* study of, de Girolamo et al. the effect of soft-focused ESWT were evaluated on a primary culture of healthy human tendon cells in adherent monolayer culture. The rationale of the study was to maintain cell-to-cell contacts and cell interactions with the ECM during ESWT, as it is a crucial point in the mechano-trasduction process. Furthermore, physical forces, such as soft-focused ESWT, may influence conformational changes in membrane proteins, such as integrines, resulting in an intra-cellular signal with a modification of gene expression and growth factors release (de Girolamo et al., 2014). At the molecular level, the authors evaluated the relationship between IL-1β and the production of MMPs, considered to be responsible for ECM degradation and tendon degeneration (Clegg et al., 2007). Both MMP-3 and 13 were not influenced by soft-focused ESW exposure, suggesting that the increased levels of IL-1β were not correlated with the ECM degradation. On the other hand, an increased expression of SCX, collagen type I genes (regulated by SCX expression), TGF-β, anti-inflammatory cytokines IL-6 and Il-10 and VEGF (stimulated by IL-6 and IL-10) was observed during the first 7 days after exposure to soft-focused ESW, although SCX transcription decreased rapidly during the first 4 days. All these results suggest that soft-focused ESWT may positively modulate the initial beneficial inflammatory phase of the tendon healing process (Visco et al., 2014) and "normalize" the anabolic activities of tendon cells.

Although the lack of sure evidence, ESWT may also be efficient in reducing calcifications in tendon structure (van der Worp et al., 2013).

Clinical Results of ESWT in Tendon's Pathology Treatment

ESWT is reported to be an effective treatment in different chronic tendon pathologies.

Shoulder

In 2014 Bannuru et al. published a systematic review on 28 Randomized Controlled Trials (RCT) comparing high-energy vs. low-energy ESWT or placebo for treatment of calcific or non-calcific tendinitis of the shoulder. The authors concluded that high-energy ESWT was significantly better than placebo in reducing pain and improving function in calcific tendinopathy, while no differences were detected between ESWT and placebo in non-calcific tendinopathy (Bannuru et al., 2014).

Huisstede et al. in 2011, in a systematic review, included 17 RCTs about ESWT vs. placebo in calcific and non-calcific rotator cuff tendinopathy. The authors concluded for strong evidence toward the best effectiveness of high-ESWT in calcific rotator cuff tendinopathy. Furthermore, no differences between the treatments were found in non-calcific rotator cuff tendinopathy (Huisstede et al., 2011). Similar results were reported in the systematic review by Harniman et al. (2004). The single studies are summarized in **Table 1**.

Patellar Tendinopathy

Van Leeuwen et al. in 2009 published a systematic review describing the results of ESWT in patellar tendinopathy, collecting seven RCTs on ESWT vs. placebo. The authors concluded on positive results using ESWT in treating patellar tendinopathy, but most of the studies had different frequency of treatments, application, and shockwave generation, energy level and method of localization (van Leeuwen et al., 2009). However, Zwerver et al. in 2011 published a RCT on 62 symptomatic athletes affected by patellar tendinopathy treated either with ESWT or placebo, concluding for no beneficial effects of ESWT (Zwerver et al., 2011). Due to these conflicting reports, further studies are needed to clarify the value of ESWT for patellar tendinopathy. Again, the most recent studies are summarized in **Table 1**.

Elbow

Buchbinder et al. in 2005 published a systematic review regarding the effectiveness and safety of ESWT for lateral elbow pain. The authors included nine trials, randomizing 1006 participants to ESWT or placebo and one trial including 93 participants randomized into ESWT or steroid injection. They concluded that steroid injections were more effective compared to ESWT (Buchbinder et al., 2005). Recently, Trentini et al. reported the results on 36 patients affected by lateral epicondylitis and treated with focal ESWT. At a mean follow-up of 24.8 months, the authors described a positive response to the treatment in 75.7% of the patients (Trentini et al., 2015). These studies outlines that, the clinical efficacy of ESWT for the treatment of medial and lateral epycondylitis is still controversial, as shown in the papers reported in **Table 1**.

Foot Pathology

In 2013 Al-Abbad et al. published a systematic review including six studies (4 RCTs) and evaluating the efficacy of ESWT for Achilles tendinitis treatment. The authors concluded that ESWT was effective for Achilles tendinopathy at a minimum 3 months'

TABLE 1 | Summary of literature studies on ESWT, Extra-corporeal Shock Waves Therapy; RSWT, radial shockwave therapy.

Authors and Year	Pathology	Number of patients	Level	Type ESWT	Follow-up	Outcomes
SHOULDER						
Loew et al., 1999	Calcific tendinitis of the shoulder	195 (80 divided in 4 groups with different regimens, 115 divided into one or two session)	II	- High-ESWT EFD: 0.30 mJ/mm^2 (high) one session—double - Low-ESWT: 0.1 mJ/mm^2(high) one session—double Control (no treatment; $n = 20$)	Not reported	The results showed energy-dependent success. With 58% of pain relief after two high-energy session
Schmitt et al., 2001	Non-calcific supraspinatus tendinitis	40	II	- High-ESWT 0.11 mJ/mm^2($n = 20$) - Sham ESWT ($n = 20$)	12 weeks	Increased function and a reduction of pain in both groups ($p <$ or $= 0.001$). The authors did not recommend ESWT for the treatment of tendinitis of supraspinatus
Speed et al., 2002a	Non-calcific supraspinatus tendinosis6	74	II	- ESWT: 0.12 mJ/mm^2(medium; $n = 34$) - ESWT: minimum: 0.04 mJ/mm^2 (low; $n = 40$)	Not reported	No significant difference between the treatments in terms of pain. The authors concluded on no benefit of ESWT in patients with non-calcific tendonitis
Haake et al., 2002	Calcific tendinitis of the supraspinatus	50	II	- ESWT: focus on calcific deposit: 0.78 mJ/mm^2(high; $n = 25$) - ESWT: focused on tuberculum majus: 0.78 mJ/mm^2 (high; $n = 25$)	1 year	Significantly better Constant and Murley score in ESWT at the calcified area under fluoroscopic control
Pan et al., 2003	Calcific tendinitis of the shoulder	63	II	- High-ES WT 2 Hz 2000 shock waves, 2 sessions, 14 days apart 0.26–0.32 mJ/mm^2($n = 33$) - TENS 3x/week 20 min for 4 weeks ($n = 30$)	6 months	Better outcomes VAS and Constant score in the ESWT group compared to TENS group
Gerdesmeyer et al., 2003	Calcific rotator cuff tendinopathy	96	II	- High-ESWT (1500 pulses 0.32 mJ/mm^2; $n = 48$) - Sham ESWT ($n = 48$)	1 year	High-ESWT and low-ESWT provided a beneficial effect on pain, function and calcifications' size. However, high-ESWT appeared to be superior compared to low-ESWT
Perlick et al., 2003	Calcific tendinitis of the shoulder	80	II	- ESWT: 0.23 mJ/mm^2(medium; $n = 40$) - ESWT: 0.42 mJ/mm^2 (high; $n = 40$)	1 year	Improvement in Constant and Murley scores. However, the disintegration of calcific deposits is dose-dependent
Peters et al., 2004	Calcific tendinosis of the shoulder	61	II	- High level ESWT: 0.44 mJ/mm^2 ($n = 31$) - Medium level ESWT: 0.15 mJ/mm^2 ($n = 30$)	6 months	ESWT in calcific tendinitis of the shoulder is very effective, without significant side effects at 0.44 mJ/mm^2
Cosentino et al., 2004	Chronic calcific tendinitis of the shoulder	135	IV	ESWT 0.03 mJ/mm^2 (4 sessions)	1 month	Improvement in the Constant and Murley score, with partial resorption of the deposits in 44.5% of patients, and complete resorption in 22.3% of patients
Krasny et al., 2005	Calcific supraspinatus tendinitis	80	II	- High-ESWT plus Ultrasound-guided needling ($n = 40$) - High-ESWT only (200 impulses followed by 2500 pulses, 0.36 mJ/mm^2($n = 40$)	4.1 months (average)	Ultrasound-guided needling in combination with high-ESWT is more effective compared to ESWT alone, with higher rates of deposits elimination, better clinical results and lower need for surgery

(Continued)

TABLE 1 | Continued

Authors and Year	Pathology	Number of patients	Level	Type ESWT	Follow-up	Outcomes
Sabeti-Aschraf et al., 2005	Calcific tendinitis of the shoulder	50	II	- ESWT: 0.08 mJ/mm^2 Point of max tenderness ($n = 25$) - ESWT: 0.08 mJ/mm^2 Point of max tenderness by computer-assisted navigation device ($n = 25$)	12 weeks	Both groups had significant improvements in the Constant and Murley score and VAS score. However, the navigation group showed better results
Moretti et al., 2005	Rotator cuff calcifying tendinitis	44	IV	Four sessions of medium-ESWT (0.11 mJ/mm^2) ESWT administered with an electromagnetic lithotripter	6 months	70% of satisfactory functional results. Disappearance of the deposits in 50% of the cases
Cacchio et al., 2006	Calcific tendinitis of the shoulder	50	II	- ESWT 4 sessions at 1-week intervals, with 25,00 pulses per session, 0.10 mJ/mm^2 ($n = 25$) - ESWT 4 sessions at 1-week intervals, total number of pulses: 25 ($n = 25$)	6 months	Better functional results in the RSWT group
Albert et al., 2007	Calcific tendinitis of the shoulder	80	II	- ESWT: max 0.45 mJ/mm^2(high; $n = 40$) - ESWT: 0.02-0.06 mJ/mm^2 (low; $n = 40$)	110 days	High-ESWT group had significant better results, but with the calcific deposit unchanged in size in the majority of patients
Hsu et al., 2008	Calcific tendinitis of the shoulder	46	II	- High-ESWT: 0.55 mJ/mm^2($n = 33$ - Sham ESWT ($n = 13$)	1 year	No significant difference between Gärtner type I and type II groups in the Constant score ($P > .05$). ESWT are effective in the treatment of calcific tendinitis with negligible complication
Rebuzzi et al., 2008	Calcific tendinitis of the supraspinatus	46	IV	- Arthroscopic extirpation ($n = 22$) - Low- ESWT ($n = 24$)	24 months	No differences in UCLA scores. ESWT have similar results compared to arthroscopy
Schofer et al., 2009	Non-calcific shoulder tendinopathy	40	II	- High-ESWT-1 0.78 mJ/mm^2($n = 20$) - High-ESWT-2 0.33 mJ/mm^2($n = 20$)	12 weeks	Statistically significant improvement in both groups, without statistically significant differences between high-ESWT and low-ESWT
Ioppolo et al., 2012	Supraspinatus Calcifying Tendinitis	46	II	- ESWT at an energy level of 0.20 mJ/mm^2 - ESWT at an energy level of 0.10 mJ/mm^2	6 months	Better results in the first group of treatment (Constant Murley Scale=CMS)
Galasso et al., 2012	Non-calcifying supraspinatus tendinopathy	20	II	- ESWT - sham control group	12 weeks	ESWT groups showed better CMS score, without any side effect
PATELLAR TENDINOPATHY						
Peers et al., 2003	Chronic patellar tendinopathy	27	III	- Surgical treatment ($n = 13$) - ESWT ($n = 14$)	6 months	ESWT showed comparable outcomes compared to surgery
Taunton and Khan, 2003	Chronic patellar tendinopathy	30	II	- ESWT ($n = 20$) - ESWT with energy-absorbing pad ($n = 10$)	Not reported	ESWT is effective in adjunction with eccentric exercises in treating patellar tendinopathy
Wang et al., 2007	Chronic patellar tendinopathy	50	II	- ESWT (0.18 mJ/mm^2 energy flux density; $n = 27$) - Conservative treatment ($n = 23$)	2-3 years	ESWT is more effective compared to conservative treatment

(Continued)

TABLE 1 | Continued

Authors and Year	Pathology	Number of patients	Level	Type ESWT	Follow-up	Outcomes
Vulpiani et al., 2007	Jumper's knee	73	IV	- ESWT (4 sessions 1500–2500 impulses,energy varying between 0.08 and 0.44 mJ/mm^2)	Not reported	Satisfactory outcomes in ESWT treatment for jumper's knee
Zwerver et al., 2010	Severe patellar tendinopathy	19	IV	Patient guided Piezo-electric, focused ESWT	3 months	Patient guided Piezo-electric ESWT without local anesthesia is a safe and well-tolerated treatment for severe patellar tendinopathy
Zwerver et al., 2011	Patellar tendinopathy in athletes	62	I	- ESWT ($n = 31$) - Sham ESWT ($n = 31$)	1 year	No benefit of ESWT over placebo in treatment of patellar tendinopathy in in-season athletes
Furia et al., 2013	Chronic patellar tendinopathy	66	III	- Radial low-ESWT ($n = 33$) - Conservative treatment ($n = 33$)	1 year	The percentage of "excellent" functional outcomes was significantly higher in the ESWT group
ELBOW PATHOLOGY						
Rompe et al., 2001	Chronic lateral epicondylitis of the elbow	30	II	- ESWT (0.16 mJ/mm^2) - ESWT (0.16 mJ/mm^2) plus cervical manual therapy	1 year	Each group showed significant improvement in the pain and functional scores. The authors concluded that ESWT may be an effective conservative treatment method for unilateral chronic tennis elbow
Maier et al., 2001	Chronic lateral tennis elbow	42	IV	ESWT	18.6 months	Good clinical performances after ESWT. Male patients performed better than female ones. In female patients, Magnetic Resonance Imaging (MRI) may predict the results of ESWT
Speed et al., 2002b	Lateral epicondylitis	75	II	- ESWT at 0.12 mJ/ mm^2 - Sham therapy	1 year	No significant difference between the groups, concluding that the placebo effect of ESWT may be considerable
Melegati et al., 2004	Lateral epicondylitis	41	II	- ESWT (Lateral tangential focusing) - ESWT (tangential focusing)	Not reported	No differences between the techniques
Furia, 2005	Chronic lateral epicondylitis	36	IV	ESWT	Not reported	77.8% were rated excellent or good on the Roles and Maudsley scale
Chung et al., 2005	Chronic lateral epicondylitis	60	II	- ESWT + stretching program - Sham therapy	1 year	No differences in clinical outcomes
Staples et al., 2008	Lateral epicondylitis	68	II	- 3 ESWT treatments - 3 treatments at a subtherapeutic dose	6 months	Little evidence in favor of ESWT in the treatment of lateral epicondylitis
Radwan et al., 2008	Resistant tennis elbow	46	II	- high- ESWT (0.22 mJ/mm^2; $n = 29$) - Percutanous tenotomy ($n = 27$)	1 year	Excellent and good results were achieved in 65.5% of patients in ESWT group and 74.1% in the percutanous group
Gunduz et al., 2012	Lateral epicondylitis	59	II	- Physical therapy - A single corticosteroid injection - ESWT	6 months	All the treatment had favorable effects on pain and grip strength in the early period

(Continued)

TABLE 1 | Continued

Authors and Year	Pathology	Number of patients	Level	Type ESWT	Follow-up	Outcomes
Lee et al., 2012	Medial and lateral epicondylitis	22	III	- ESWT group (0.06–0.12 mJ/mm^2; $n = 12$) - local steroid injection group ($n = 10$)	8 weeks	Both the treatments were effective for medial and lateral epicondylitis
Notarnicola et al., 2014	Epicondylitis	26	IV	ESWT	Not reported	Progressive improvement in pain during the follow-up, with decrease in grip strength, especially in the dominant limb
Trentini et al., 2015	Lateral epicondylitis	36	IV	Focused ESWT	24.8 months	75.7% of positive response. Focal ESWT is a valuable and safe solution in case of lateral epicondylitis, both in newly diagnosed and previously treated cases
FOOT PATHOLOGY						
Furia, 2006	Insertional Achilles tendinopathy	88	II	- ESWT group; 0.21 mJ/mm^2; total energy flux density, 604 mJ/mm^2 ($n = 35$) - Non-operative therapy ($n = 33$)	1 year	Better Roles and Maudsley results in the ESWT group. NO differences if a local anesthesia was performed or not before the ESWT session
Rasmussen et al., 2008	Chronic Achilles tendinopathy	48	II	- active ESWT - sham ESWT	12 weeks	Better results in the active ESWT group
Vulpiani et al., 2009	Achilles tendinopathy	115	IV	ESWT (0.08 and 0.40 mJ/mm^2)	1 year	76% of satisfactory results at the last follow-up
Saxena et al., 2011	Achilles tendinopathy	74	IV	ESWT	1 year	74.8% of patients improved 1 year after surgery, with significant improvement of the Roles and Maudsley score
Kim et al., 2015	Plantar fasciitis	10	IV	ESWT	6 months	Decreased plantar fascia thickness, spasticity, and pain and increased gait ability after ESWT

follow-up (Al-Abbad and Simon, 2013). Other studies pointed out similar conclusions as shown in **Table 1**.

Recently, Kim et al. analyzed the efficacy of ESWT also for plantar fasciitis in stroke patients, reporting on reduced tension in the plantar fascia, and observing pain relief and improved gait ability (Kim et al., 2015; **Table 1**). Their results are similar to other recent studies (Park et al., 2014; Yin et al., 2014; Gollwitzer et al., 2015; Konjen et al., 2015; Mardani-Kivi et al., 2015), and allows for considering ESW a precious therapeutic modality for treating acute or recalcitrant plantar fasciitis with acceptable success rate ranging from 50% to 80%, at least at a short term follow-up.

PULSED ELECTROMAGNETIC FIELDS (PEMF) FOR TENDON'S PATHOLOGY

Pulsed Electromagnetic Fields (PEMF) Definition and Mechanism of Action

PEMF are characterized by frequencies at the low end of the electromagnetic spectrum, ranging between 6 and 500 Hz (Bassett, 1989). Another feature of PEMF waveforms is the rate of change: higher rate of changes (Tesla/seconds) are able to induce biological currents in the tissue, with peculiar biological effects (Juutilainen and Lang, 1997). Furthermore, it was demonstrated that low-frequency fields are non-ionizing and athermal (Rubik, 1997).

Different types of waveforms were associated to PEMF: asymmetric, biphasic, sinusoidal, quasi-rectangular, or quasi-triangular in shape (Bassett, 1989). In 1979, the Food and Drug Administration (FDA) approved both quasi-rectangular and quasi-triangular PEMF as safe and effective for the treatment of fractures and their sequelae (Bassett, 1989).

There are two methods in which PEMF can be applied to biological tissues: capacitive or inductive coupling. Capacitive coupling does not involve any contact with the body. However, in direct capacitive coupling, an electrode has to be placed on the skin of the opposite side (Trock, 2000). On contrary inductive coupling does not require the electrodes to be in direct contact with the skin, because the magnetic field produces an electric field that, in turn, produces a current in the conductive tissues of the body (Stiller et al., 1992; Trock, 2000).

Similarly to ESW, PEMF are physical stimuli that produce membrane disturbances and activation of multiple intracellular pathways. Indeed, formation of lipidic "nanopores" in the plasma membrane following PEMF exposure may explain the conduction of ions into the cell from the extracellular space, specifically Calcium ions (Ca). Furthermore, a direct effect of PEMF on phospholipids within the plasma membrane has been postulated, with a subsequent production of several second messengers, initiating multiple intracellular signal transduction pathways, as well as a further activation of protein kinase C (Semenov et al., 2013; Tolstykh et al., 2013).

Nevertheless, PEMFs have been recently connected to other cell activation pathways. In particular, the ligand-independent activation of epidermal growth factor receptor (EGFR) and other members of the receptor tyrosine kinase family were observed, with subsequent stimulation of intracellular signaling as the MAPK (mitogen-activated protein kinases)/ERK (extracellular signal-regulated kinases) pathway, with subsequently activation of intracellular mitogenic pathway (Wolf-Goldberg et al., 2013).

Recent studies outlined the expression of canonical Wnt signaling proteins (Wnt1/ lipoprotein receptor-related protein 5 LRP5/beta-catenin) in cell derived from a mesenchymal lineage and exposed to PEMF (Jing et al., 2013; Zhou et al., 2015). At this regard, the presence of beta-catenin seems particularly important with respect to other settings, in which this protein is linked to cell plasticity and proliferation, as in the superficial zone of articular cartilage (Yasuhara et al., 2011; Marmotti et al., 2013).

Finally, the exposure to PEMF induces early up-regulation of adenosine receptor A_{2A} and A_3. Adenosine receptor A_{2A} and A_3 are able to reduce PGE2 and pro-inflammatory cytokine IL-6 and IL-8 release and to inhibit the activation of transcription factor NF-kB, a key regulator of inflammatory responses (Vincenzi et al., 2013), as well as to positively interfere in several cell activities as cell proliferation (Varani et al., 2008).

Taken together, all these observations suggest a possible role of PEMF for "tenocyte activation" (Dingemanse et al., 2014; de Girolamo et al., 2015). This may be achieved by two main mechanisms: (i) limiting the catabolic effects of pro-inflammatory cytokines such as IL-1, IL-6, and IL-8 and (ii) increasing ECM production, cytokine release and cell proliferation (De Mattei et al., 2003; Fassina et al., 2006; Ongaro et al., 2012; de Girolamo et al., 2013). Recent studies of de Girolamo et al. (de Girolamo et al., 2013, 2015) demonstrated that human tendon cells proliferation was enhanced after PEMF treatment, and in particular, that a 1.5 mT-PEMF treatment was able to up-regulate SCX, VEGF-A, and COL1A1 gene expression. Moreover, the treated tendon cells showed, after 2 days, a higher release of IL-6, IL-10, and TGF-β. These effects are essential for tendon metabolism, inducing increased elastin and fibronectin production, increased cell proliferation and neo-angiogenesis.

Clinical Results of PEMF in Tendon's Pathology Treatment

Despite the lack of recent literature sustaining a long-term positive effect of PEMF in treating shoulder and elbow tendon disorders (Uzunca et al., 2007; Bisset et al., 2011; Dingemanse et al., 2014), a positive effect of PEMF in reducing lateral epicondylitis pain was described at a short term follow-up (3 months). Moreover, several reports back in the 80's proposed a putative role in the treatment of rotator cuff disease and lateral epicondylitis (Binder et al., 1984; Devereaux et al., 1985). A very recent study by Osti et al. described a possible role of PEMF after rotator cuff repair as an adjuvant treatment, in order to reduce local inflammation, post-operative joint swelling and stiffness, and recovery time, as well as to induce pain relief. A significant short term (up to 5 months) positive effect was observed in PEMF treated patients, but the authors did not observe any clinical and functional improvement at a longer (2 years) follow-up (Osti et al., 2015).

Some suggestions for the use of PEMF for treating human Achilles tendon pathologies come only from preclinical animal studies. Strauch et al. in 2006 analyzed the effect of PEMF on the biomechanical strength of rat Achilles' tendons at 3 weeks after transection and surgical repair, with an increase in tensile strength of up to 69% 3 weeks after the surgery (Strauch et al., 2006). Previously, Lee et al. in 1997 analyzed the possible role of pulsed magnetic fields (PMF) and (PEMF) in the healing process of Achilles tendon inflammation in the rat (Lee et al., 1997).

CONCLUSION

Mechanical stimulation by means of ESW and PEMF seems to be favorable for tendon regeneration in preclinical and *in vitro* studies. Indeed, a beneficial effect has been demonstrated on tendon resident cells at a cellular level. Increased cell proliferation and the production of the immuno-regulatory cytokines and growth and angiogenic factors was observed *in vitro*, consistent with an overall "tenocyte activation" favoring tendon healing process. Conversely, there is still a lack of strong evidences on ESW and PEMF when dealing with clinical settings. The efficacy of ESWT is demonstrated for the treatment of calcific rotator cuff tendinopathy, while it is still debated its role in the treatment of other tendon disorders, such as patellar tendinopathy and lateral epicondylitis. PEMF seem to exert positive clinical effects toward shoulder and elbow tendon disorders only at a short term follow up. While basic science research continues to show encouraging results, further *in vivo* human studies are undoubtedly necessary to confirm the clinical efficacy of mechanical stimulation by means of PEMF and ESW for the treatment of tendon disorders.

AUTHOR CONTRIBUTIONS

FR Substantial contributions to the conception or design of the work, drafting the work, acquisition, analysis, or interpretation of data for the work. DB Substantial contributions to the conception or design of the work, revising the work. AM Substantial contributions to the conception or design of the work, revising the work. UC Substantial contributions to the conception or design of the work, revising the work. RR Revising the work, Final approval of the version to be published.

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frontiers in Aging Neuroscience

MINI REVIEW
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Nanoparticles for Tendon Healing and Regeneration: Literature Review

Paolo D. Parchi[1], Orazio Vittorio[2,3], Lorenzo Andreani[1], Pietro Battistini[1], Nicola Piolanti[1], Stefano Marchetti[1], Andrea Poggetti[1] and Michele Lisanti[1]*

[1] First Orthopaedic Division, Department of Translational Research and New Technology in Medicine and Surgery, University of Pisa, Pisa, Italy, [2] Lowy Cancer Research Centre, Children's Cancer Institute Australia, UNSW Australia, Sydney, NSW, Australia, [3] Australian Centre for NanoMedicine, UNSW Australia, Sydney, NSW, Australia

Tendon injuries are commonly met in the emergency department. Unfortunately, tendon tissue has limited regeneration potential and usually the consequent formation of scar tissue causes inferior mechanical properties. Nanoparticles could be used in different way to improve tendon healing and regeneration, ranging from scaffolds manufacturing (increasing the strength and endurance or anti-adhesions, anti-microbial, and anti-inflammatory properties) to gene therapy. This paper aims to summarize the most relevant studies showing the potential application of nanoparticles for tendon tissue regeneration.

Keywords: nanoparticles, tendon injuries, scaffold, silver nanoparticles, gold nanoparticles

INTRODUCTION

Tendon injuries could be caused by trauma, but most of them are the result of gradual wear and tear of the tendon from overuse or aging (Thomopoulos et al., 2015). A tendon injury may seem to happen suddenly, but usually it is the result of many tiny tears to the tendon that have happened over time (Molloy and Wood, 2009). Tendons show limited regeneration potential and, most of the time there is scar tissue formation which causes inferior mechanical properties (Molloy and Wood, 2009). Clinically, the healing of Achilles tendon usually takes 4–8 weeks; however, a full return to sport activities is only recommended after a long span of 4–12 months.

To overcome these problems new strategies based on stem cell transplantation and growth factors have been proposed (Sahni et al., 2014; Lui, 2015). The ability of several growth factors to improve the healing response and decrease scar formation is described in different preclinical studies (Sahni et al., 2014; Lui, 2015). Besides the application of growth factors and stem cell transplantation, innovative research focuses on the development of nano-structured scaffolds to improve the healing response in tendon injuries (Oragui et al., 2012).

Structurally tendons and associated extracellular matrices are composed of nanostructured materials. For this reason, in the last years there was a growing interest to develop novel nano-materials for tendon regeneration. Nanotechnology is the precise placement, measurement, manipulation, and modeling of matter that consists of 4–400 atoms. The range below 100 nm is important because the classic law of physics change, resulting in novel physical properties that allow researchers to produce new materials with exact properties, such as size and strength beyond conventional limits. Nanomaterials have been proposed to improve tendon regeneration and to decrease the form of scar tissue and fibrous adhesions.

Nanoparticles (NPs) are a material (0-D), in which almost a dimension is <100 nm. NPs represent a bridge between the material of conventional size and structures at the atomic level. Indeed, biomedical engineering studied for decades the important characteristics (size, magnetizability, functionalization) to be exploited to create new treatment strategies in different

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Edited by:
Cesare Faldini,
Istituto Ortopedico Rizzoli, Italy

Reviewed by:
Nevena Divac,
University of Belgrade, Serbia
Jingwen Niu,
Temple University, USA

****Correspondence:***
Paolo D. Parchi
paolo.parchi@unipi.it

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medical areas (oncology, nerve regeneration and tissue regeneration, infettivology, radiology, etc.).

The aim of this paper is to review and discuss the current advances in nanoparticles applications for tendon tissue regeneration.

The nanoparticles could be exploited in several ways: in the manufacture of scaffolds, increasing the strength, and endurance or anti-adhesions, anti-microbial, and anti-inflammatory properties; as a carrier in gene therapy, for an anti-adhesions and anti-inflammatory activity; using directly their anti-inflammatory properties and relationship with the extracellular matrix (ECM) and cell surfaces; aid in the iontophoresis and phonophoresis using anti-inflammatory properties; labeling and tracking stem-cells with MRI.

NANOPARTICLES FOR DIRECT MEDICAL APPLICATIONS

An important application of nanoparticles is represented by their use as nano-delivery system for the treatment of tendinitis. In particular, nanoparticles have been used in Iontophoresis and phonophoresis, techniques capable of enhancing drug penetration through the skin. Phonophoresis uses ultrasound waves to deliver drugs through the skin, and iontophoresis uses low level electric current. Both techniques are usually used to treat inflammatory conditions such as tendonitis. Dohenert et al. studied the possibility to improve the drug transport during Iontophoresis and phonophoresis using gold nanoparticles (GNPs) as drug nanotrasporter (Dohnert et al., 2012, 2015). They studied the use of GNPs functionalized with Diclofenac diethylammonium in the treatment of a tendinopathy in animal model. The results of this study showed that the use of GNPs is associated with a strong a down-modulation of the inflammatory response (reduction IL-1β and TNF-α) which resulted by an improved of drug delivery to the site of injury (Dohnert et al., 2012, 2015). The authors underline that GNPs exerted anti-inflammatory and synergistic action: enabling the transport of the drug used and enhancing the therapeutic role of iontophoresis and phonophoresis (Dohnert et al., 2012, 2015).

NANOPARTICLES FOR microRNA DELIVERY

The delivery of nucleic acid *in vivo* has been preferentially performed by using viral vectors with some concerns about the safety of these procedures in patients (Vannucci et al., 2013). Nanotechnology has developed several nanoparticles to be used for gene therapy to replace the viral vectors and avoid their side effects (Raffa et al., 2011). Zhou et al. evaluated the use of nanoparticles as non-viral vector for gene therapy to prevent peritendineus adhesion formation (Zhou et al., 2013). The authors reported that the miRNAs reducing the expression of TGF-b1 (induce fibrotic changes and adhesion formations in the tissues like tendons) were inserted into the plasmid, and then the generated plasmids were loaded into PEI-polylactic-co-glycolic acid (PLGA) nanoparticles for preventing peritendinous adhesion (Zhou et al., 2013). Interestingly, they performed their study *in vitro,* using a culture of primary tenocites from flexor digitorum profundus tendon of Leghorn chicken, and *in vivo* by using the same type of tendon. The analysis of the data obtained by using electron microscopy imaging, molecular biology techniques, and biomechanical tests, underline the potential of PLGA nanoparticles as an innovative and efficient agent for gene delivery in tendon. In fact, the transfection of miRNA by PLGA nanoparticles resulted in an inhibition of TGF-b1 expressions, which in turn induced the repair of the tendon (Zhou et al., 2013). The results of this study showed the strength of the treated tendons was lower of than in the control group due to the down regulation of cells migration, proliferation, adherence, apoptosis, and of the secretion of ECM related to the inhibition og TGF-b1 (Zhou et al., 2013). The authors concluded that the purely inhibition of the expression of TGF could not achieve the desired healing effect of injured tendon. It was hyphosesized that a better results on tendon repair could be achieved by combining TGF-b1 miRNA plasmid with other miRNA for other growth factor genes, to be delivered simultaneously by nanoparticles (Zhou et al., 2013).

Brevet et al. (2014) performed functionalization of Mesoporous silica nanoparticles (MSN) with L-histidine and show a better efficiency of histidine-functionalized MSN in transfecting cells than imidazole- or amino- functionalized MSN (Brevet et al., 2014); the study was conducted *in vitro* and *in vivo* (Mice Achilles tendon; Brevet et al., 2014). The results confirmed a good gene delivery efficiency *in vitro*, but it was lower *in vivo* (Brevet et al., 2014). However, additional studies are working in the modification of MSN to increase their potential as delivery system for nucleic acid for the treatment of tendon injuries.

NANOPARTICLES FOR SCAFFOLD MANUFACTURING

Biological or synthetic scaffolds has been introduced to give a mechanical support to the tendon during the healing process (Longo et al., 2012). In regenerative medicine scaffolds are often used in combination with growth factors and stem cells to have a structural (mechanical) and biological support to the tissue healing. In tissue engineering the scaffolds could also functionalization using nanoparticles to give them new chemical and physical properties.

Karthikeyan et al. (2011) developed a system to produce functional biofibers containing silk fibers (SF) coated with chitosan and impregnated with silver nanoparticles (Ag–C–SF). Chitosan [poly-b-(1-4)-D-glucosamine] is a sustainable, biocompatible, biodegradable, and antimicrobial polysaccharide of great relevance in many fields of application. They tested the fibers with microbiological assays, Scanning electron microscopy (SEM), infrared spectroscopy, AFM studies and thermo gravimetric analysis (Karthikeyan et al., 2011). The results shown antimicrobial activity (nanoparticles enter into bacteria, inhibit the ATP Synthesis and denature DNA, and blocking the respiratory chain) and increased thermal stability (Karthikeyan et al., 2011). The authors emphasize that this fiber

might be a promising material in wound healing and tendon reconstruction (Karthikeyan et al., 2011).

Liu et al. (2013a) studied the behavior of silver nanoparticles (AgNPs) directly electro spun into biodegradable poly (L-lactide; PLLA) fibrous membrane (Liu et al., 2013a). Electro spun fibrous membranes are attractive barriers for tissue separation and drug delivery to get drug-loaded materials with lengthened releasing time because of their large surface area and controlled porous structure. The transmission electron microscopy (TEM) micrographs of the fibers showed that the AgNPs were successfully electro spun into the PLLA fibers in different contents with ability of Ag ion release (Liu et al., 2013a). The anti-proliferation effect of the AgNP-loaded PLLA fibrous membranes was observed on fibroblasts. Furthermore, no cytotoxicity was detected (Liu et al., 2013a). The broad-spectrum topical antimicrobial activity of AgNP-loaded PLLA fibrous membranes on *S. epidermidis*, *S. aureus*, and *P. aeruginosa* was certificated (Liu et al., 2013a). These properties make silver ions just suitable for prompting anti-adhesion treatment and simultaneously early prevention of infection. Moreover, although cell proliferation on the surfaces of AgNP-loaded PLLA fibrous membranes was worse than on the surface of PLLA fibrous membrane, the traditional negative effect that prevents cell proliferation was now treated as the positive effect that inhibits adhesion formation (Liu et al., 2013a). In fact, the prevention of bacterial adhesion should assist in reduction of device associated infection. This *in vitro* study showed that the AgNP-loaded PLLA fibrous membranes have a significant effect of preventing cell adhesion and proliferation without significant cytotoxicity (Liu et al., 2013a).

Chen et al. (2014) also used an electro spun fibrous membrane loaded with AgNPs (Chen et al., 2014). They prepared a combination of Ibuprofen (IBU) and Ag for decreasing kidney and liver damages caused by high dose of Ag, while maintaining good anti-adhesion effect (Chen et al., 2014). Also in this *in vitro* study, it was demonstrated that the electro spun Ag/IBU-loaded PLLA fibrous membrane not only prevented cell adhesion and proliferation but also reduced bacterial infection through its stable release of silver ions and IBU (Chen et al., 2014).

In another study of Liu et al. (2013b), PLLA was loaded with growth factors (heparin binding site of basic fibroblast growth factor [bFGFs]; Liu et al., 2013b). bFGF has been shown to stimulate angiogenesis, cellular differentiation, migration, proliferation, and matrix synthesis *in vivo* and *in vitro* in a variety of tendons. Growth factors could be used to promote tendon cells differentiation but one of the main limits relate to their use in the clinical practice are due to their poor biodisponibility *in vivo*. The possible way to overcome this problem is to use the growth factors in combination with scaffolds.

In this study, one preformulated dextran glassy nanoparticles (DGNs) loaded with bFGF were electro spun into a PLLA copolymer fiber to secure the biological activity of bFGF in a sustained manner and thus to enhance tendon healing and simultaneously prevent peritendinous adhesion (Liu et al., 2013b). Their analysis of data *in vitro* and *in vivo* (Achilles tendon of Spague-Dawley rat) shows a good protein encapsulation efficiency of the bFGF/DGNs-PLLA membrane with a release kinetic of nearly 30 days; the bFGF/ DGNs-loaded PLLA fibrous membrane can release bFGF sustainably and secure the bioactivity of bFGF (Liu et al., 2013b). The authors concluded that bFGF/DGNs-loaded PLLA membrane can protect the bioactivity of bFGF in a sustained manner for promotion of tendon healing and simultaneous adhesion prevention (Liu et al., 2013b).

Recently, there was a growing interest regarding the use of Nano cellulose materials for scaffolds design. He et al. (2014) used cellulose Nano crystals (CNCs) as reinforcement polymer Nano composites (He et al., 2014). The authors evaluated the possibility of fabricating uniaxially aligned electro spun nanofiber nonwovens from cotton cellulose and their potential application in tissue engineering (He et al., 2014). Morphology investigation from SEM images indicated that most of the obtained cellulose nanofibers were aligned and a more uniform morphology can be obtained with the incorporation of CNCs. Cell culture experiments demonstrated that the electro spun cellulose reinforced with CNCs promote fibroblast cells attachment and proliferation in the entire scaffold (He et al., 2014).

NANOPARTICLES FOR TENDON HEALING (ANTI-MICROBIAL EFFECT, ANTI-ADHESION EFFECT AND EXTRACELLULAR MATRIX COMPOSITION MODULATION)

Silver nanoparticles have also been recognized as antimicrobial agents because they inhibit ATP Synthesis in the microorganism, denature the DNA and block the respiratory chain (Klueh et al., 2000; Kumar et al., 2005; Morones et al., 2005). AgNPs not only exert anti-microbial effect, but are also capable of accelerating burn wound healing due their antiflogistic effects (Klueh et al., 2000; Kumar et al., 2005; Morones et al., 2005).

Kwan et al. (2014) investigated the effects of silver nanoparticles in the tendon healing process *in vitro* and *in vivo* using Achilles Sprague Dawnley rat (Kwan et al., 2014). In the *in vitro* evaluation the authors showed that the silver nanoparticles promote the proliferation of primary tenocytes to AgNPs and the production of ECM components (Kwan et al., 2014). In the *in vivo* evaluation the tensile tests showed that the tensile modulus of the nanoparticles-treated group was significantly better in comparison to the control group but a quite less than the normal tendon (Kwan et al., 2014). The silver nanoparticles accelerated the tendon healing process and modulate the ECM composition (more and better quality collagen fibrils). The results of this study showed that silver nanoparticles have a positive effect in Achilles tendon healing, through boosting cell proliferation and stimulating the production of collagen and proteoglycans (Kwan et al., 2014). The silver nanoparticles also showed an antiflogistic effect reducing the formation of scar tissue and adhesions (Kwan et al., 2014).

Empson et al. (2014) explored the *in vitro* biomechanical and cellular response of the therapy with nanoparticles for damaged connective tissues (Empson et al., 2014). They hypothesized that

the controlled and localized injection of biocompatible NPs into damaged connective tissues would enhance matrix mechanical properties, evidenced by increased stiffness, and yield strength (Empson et al., 2014). The effects of NPs, namely, single-walled CNHs and CNCs, on damaged connective tissue mechanical properties were examined. In the study conducted by Empson et al. the authors evaluated the effects of the use of nanoparticles CNHs and CNCs using different types of connective tissues the porcine skin, that mimics the mechanical and biological properties of human ligaments and tendons, and the porcine tendon as a model of the treatment of target tissues (Empson et al., 2014). They analyzed data with atomic force microscopy (ATM), dynamic light scattering (DLM), cell cultures and mechanical tests. Authors underline that the results presented in this study show the feasibility of using CNHs and CNCs to locally reinforce damaged connective tissues (Empson et al., 2014).

Recent works have also shown the ability of nanoparticles (NPs) to modulate the cellular responses (Fisher et al., 2010; Kwan et al., 2011) and the ECM mechanical properties (Bhattacharyya et al., 2006; Li et al., 2009).

In addition to triggering a cellular response, NPs have also been shown to enhance the mechanical properties of natural polymer matrices; examples include chitosan, regenerated cellulose, and decellularized porcine diaphragm tendon (Qi et al., 2009; Deeken et al., 2011).

NANOPARTICLES AND MAGNETIC RESONANCE IMAGING (MRI)

Nanoparticles can be used to improve the quality of MR imaging. Yang et al. (2013) studied the feasibility of labeling tendon stem cells (TSCs) with super-paramagnetic iron oxide (SPIO) nanoparticles to track TSCs *in vivo* using MRI (Yang et al., 2013). Although bone marrow mesenchymal stem cells have been used to repair injured tendons, they frequently cause bone formation in healed tendons. Therefore, TSCs may be more suitable than BMSCs to effectively repair acute and chronic tendon or ligament injuries.

The authors conducted an *in vitro in vivo* (rabbit model) study in which the TSCs has been incubated with appropriate concentration of SPIO (Yang et al., 2013). The results of this study showed that the TSCs maintain their biological characteristics and they could be traceable by MRI. The mechanism of SPIO cellular uptake is likely through receptor-mediated endocytosis and therefore maintaining the cells endocytosis capabilities is very important (Yang et al., 2013). In general, higher the SPIO dosag, longer labeling efficiency will be maintained. However, high doses of SPIO may have detrimental effects on cells, such as reduction in cell viability. Therefore, the balance between labeling efficiency, cell viability, and MRI signal should be considered when labeling cells. Comparing the results from using 100, 50, and 25 lg Fe/mL of SPIO, they found that high labeling efficiency without causing apparent changes to cell morphology can be achieved at 50 lg Fe/mL of SPIO concentrations (Yang et al., 2013). The authors underline that one limitation of this study is that MRI does not have sufficient resolution to detect individual cells and differentiate the "status" of labeled cells (Yang et al., 2013). The authors concluded that SPIO labeling did not change TSC viability, proliferation, or stemness, and that labeled cells could be tracked by MRI in a rabbit tendon injury model (Yang et al., 2013).

CONCLUSION

Structurally tendons and associated extracellular matrices are composed of nanostructured materials. For this reason, in the last years there was a growing interest on new approaches for tendon regeneration based on nano-materials. Nanoparticles (NPs) in which almost a dimension is <100 nm, represent a bridge between the conventional size materials, that are actually used in orthopedic surgery, and the atomic level tendon structures. In this review, we have presented some of the papers describing how nanoparticles could play an important role in tendon healing: labeling TSCs, working as carrier for gene therapy and for drug delivery, allowing the fabrication of a new generation of bioactive scaffolds and modulating the cellular and the ECM response. An important point to be addressed before the translation of nanomaterials to the clinic, is to assess the safety of these nanoparticles in the human body when they are used alone or as a component of an implantable devices.

It is clear that more work has to be done to find the best approach for tendons regeneration. Nanotechnology has a big potential in this field. This has become a multidisciplinary challenge and we believe that collaboration between orthopedic surgeons and experts in nanotechnology can make the difference in this challenge.

AUTHOR CONTRIBUTIONS

PP: online research and abstract selection, coordination between authors and general paper composition, Abstract, Introduction, manuscript revision process and manuscript preprint editing. OV: online research and abstract selection, coordination between authors and general paper composition, Conclusion. LA: Nanoparticles for direct medical applications. PB: Nanoparticles for microRNA delivery. NP: Nanoparticles for scaffold manufacturing. SM: Nanoparticles and extracellular matrix (ECM) and cellular surface modulation. AP: Nanoparticles and magnetic resonance imaging (MRI). ML: Supervision.

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REVIEW
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The Role of Detraining in Tendon Mechanobiology

Antonio Frizziero[1], Francesca Salamanna[2], Elena Della Bella[3,4], Filippo Vittadini[1], Giuseppe Gasparre[1], Nicolò Nicoli Aldini[2,3], Stefano Masiero[1] and Milena Fini[2,3]*

[1] Department of Physical and Rehabilitation Medicine, University of Padua, Padua, Italy, [2] Laboratory of Biocompatibility, Technological Innovations and Advanced Therapies, RIT Department, Rizzoli Orthopedic Institute, Bologna, Italy, [3] Laboratory of Preclinical and Surgical Studies, Rizzoli Orthopedic Institute, Bologna, Italy, [4] Department of Experimental, Diagnostic and Specialty Medicine, University of Bologna, Bologna, Italy

Introduction: Several conditions such as training, aging, estrogen deficiency and drugs could affect the biological and anatomo-physiological characteristics of the tendon. Additionally, recent preclinical and clinical studies examined the effect of detraining on tendon, showing alterations in its structure and morphology and in tenocyte mechanobiology. However, few data evaluated the importance that cessation of training might have on tendon. Basically, we do not fully understand how tendons react to a phase of training followed by sudden detraining. Therefore, within this review, we summarize the studies where tendon detraining was examined.

Materials and Methods: A descriptive systematic literature review was carried out by searching three databases (PubMed, Scopus and Web of Knowledge) on tendon detraining. Original articles in English from 2000 to 2015 were included. In addition, the search was extended to the reference lists of the selected articles. A public reference manager (www.mendeley.com) was adopted to remove duplicate articles.

Results: An initial literature search yielded 134 references (www.pubmed.org: 53; www.scopus.com: 11; www.webofknowledge.com: 70). Fifteen publications were extracted based on the title for further analysis by two independent reviewers. Abstracts and complete articles were after that reviewed to evaluate if they met inclusion criteria.

Conclusions: The revised literature comprised four clinical studies and an *in vitro* and three *in vivo* reports. Overall, the results showed that tendon structure and properties after detraining are compromised, with an alteration in the tissue structural organization and mechanical properties. Clinical studies usually showed a lesser extent of tendon alterations, probably because preclinical studies permit an in-depth evaluation of tendon modifications, which is hard to perform in human subjects. In conclusion, after a period of sudden detraining (e.g., after an injury), physical activity should be taken with caution, following a targeted rehabilitation program. However, further research should be performed to fully understand the effect of sudden detraining on tendons.

Keywords: tendon, tenocyte, detraining, sudden detraining, systematic literature review

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Edited by:
Paolo Bonaldo, University of Padova, Italy

Reviewed by:
Francesco Oliva, University of Rome Tor Vergata, Italy
Cosimo Costantino, University of Parma, Italy

****Correspondence:***
Francesca Salamanna
francesca.salamanna@ior.it

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INTRODUCTION

Tendons are a specialized tissues that join muscle to bone and are composed by extracellular collagen fibers arranged in regular arrays (Aslan et al., 2008). This mechanosensitive tissue shows detailed mechanical properties that allow it to adapt and respond to loading transmitted by muscles (Fang and Lake, 2015). This load transfer provide the principal mechanical stimulus for tendon cells (Kondratko-Mittnacht et al., 2015). These tensile loads are diverted to tendon cells through different matrix compartments and components. At cellular level, by various transmembrane structures and pathways, they are transduced from the exterior to intracellular biochemical responses (Kondratko-Mittnacht et al., 2015; Maeda and Ohashi, 2015).

While physiologic loads are required to maintain tendon homeostasis, (Galloway et al., 2013) unusual loading could direct to tendon injury, either through an acute traumatic injury or chronic, degenerative process (i.e., tendinopathy) resulting from an increase of microdamages and an altered cell/matrix response (Arnoczky et al., 2007; Magnusson et al., 2010). Histopathologicaly, tendinopathy is a unsuccessful healing response, represented by altered tenocytes proliferation, disruption and impaired organization of collagen fibers, increase in non collagenous matrix and neovascularization (Maffulli et al., 2011). In the chronic stage of tendinopathy, inflammation is absent or minimal, nevertheless it could play a role only in the initiation, but not in the propagation and progression, of the disease process (Maffulli et al., 2010). Even if tendinopathies also comprise conditions of damage to the tendon without symptoms, these pathologies frequently occur with pain in the injured tendon, which is accentuated or appears during palpation of the affected area or during active and passive movements involving the tendon (Franceschi et al., 2014). Tendon injury may not only lead in the lack of mobility or irregular joint kinematics, but could also result in damages to tissues adjacent to the joint. Muscle atrophy subsequent to tendon rupture is a frequent complication found by physicians and orthopedic surgeons. This condition proves significantly weaker musculature resulting in unfavorable functional consequences, with a consequent reduction in muscle force generation (Sandri, 2008; Zhang et al., 2013). Despite previous studies showed complete histological and biochemical characteristics of tendons rupture and some of these have been included into the clinical scenario, little is known concerning the mechanical response of muscles to tendon injury (Jamali et al., 2000; Derwin et al., 2006; Sandri, 2008; Charvet et al., 2012; Zhang et al., 2013). However, recently Zhang et al. (2013) demonstrated that tendon rupture has a supplementary influence on muscle biomechanics in comparison to disuse.

Due to their poor healing ability, tendon injuries represent an increasing problem in orthopedics as physicians are faced with a growing demand in sports and recreation and in the aging population (Kaux et al., 2011). Thus, primary disorders of tendons are a widely distributed clinical problem in society and hospital evidence and statistical data suggest that some tendons are more susceptible to pathology than others; these are the rotator cuff, Achilles tibialis posterior and patellar tendons. Although there are no specific figures in relation to tendon disease, several studies show that 16% of the population is affected from tendon pain (Urwin et al., 1998) and this rises to 21% when the statistics shift to elderly hospitals and community populations (Chard et al., 1991; Urwin et al., 1998). These numbers supplementary enhance in the sports community, in fact it was reported that 30–50% of all sporting injuries involve tendons (Kannus and Natri, 1997). Ordinarily, the major conditions affecting tendons are tendinitis and tendinosis; the first assumed to be accompanied by inflammation and pain, whereas the second can be caused by tendinous degeneration (Maffulli et al., 1998). It is assumed that these conditions are seldom spontaneous (Gibson, 1998) and are not caused by single factors. Rather, they are the end result of a variety of pathological processes (Riley, 2004; Rees et al., 2006) which can ultimately lead to the main clinical problem: loss of tissue integrity with full or partial tendon rupture.

Many intrinsic and extrinsic factors such as aging, gender, anatomical variants, obesity, systemic diseases, estrogen deficiency, drugs, sporting activities, physical loading, occupation, and environmental conditions could affect the biological and anatomo-physiological characteristics of the tendon (Nakama et al., 2005; Holmes and Lin, 2006; Torricelli et al., 2006, 2013; Frey and Zamora, 2007; Franchi et al., 2013; Frizziero et al., 2013, 2014; Malliaras et al., 2013; Moerch et al., 2013; Abate, 2014; Berardi et al., 2014; Boivin et al., 2014; Galdiero et al., 2014; Hast et al., 2014; Oliva et al., 2014a,b; Snedeker and Gautieri, 2014; Sandberg et al., 2015). Thus, over the past decade, tendon and tenocyte adaptations in relation to immobilization, training, aging and medications have been the center of an growing number of studies (Maffulli et al., 2003; Sharma and Maffulli, 2005; Torricelli et al., 2006, 2013; Stanley et al., 2008).

While proper mechanical loads at physiological levels are typically helpful to tendons in terms of enhancing its mechanical properties, recent preclinical and clinical studies examining the effect of detraining on tendon, showed alterations in its structure and morphology and in tenocyte mechanobiology. However, there is a paucity of data that evaluated the impact that detraining may have on tendon. Thus, it has not yet been understood how tendons behave to a period of training followed by cessation of training. Nevertheless, to guide rehabilitation and/or athletic programs it is necessary to elucidate tendon adaptation after sudden detraining. Therefore, within this descriptive systematic literature review, we summarize the studies where tendon detraining was examined.

MATERIALS AND METHODS

Descriptive Literature Review

According to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) a systematic search was carried out for this descriptive literature review (see **Figure 1** for details) in three databases (www.pubmed.org,

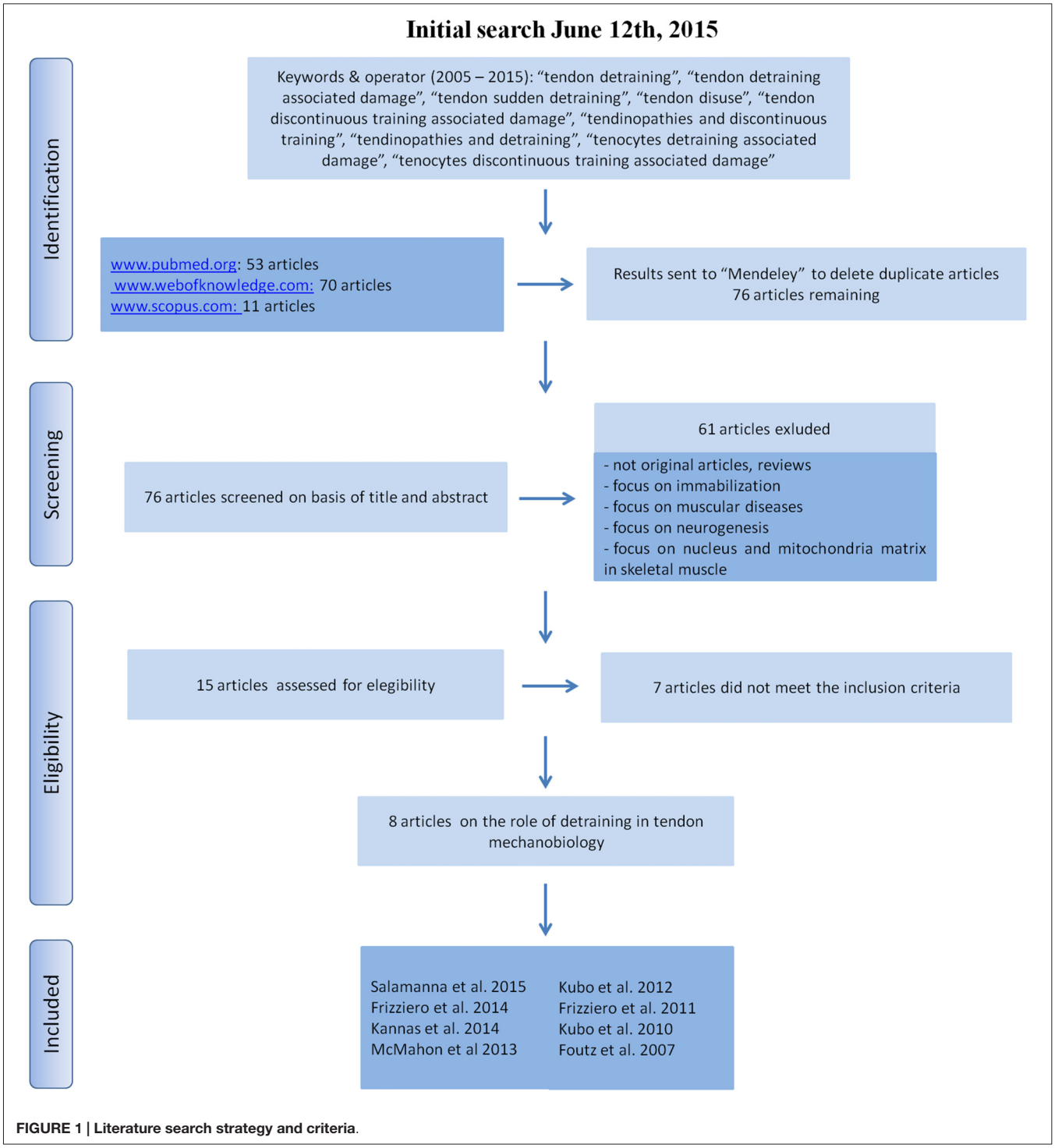

FIGURE 1 | Literature search strategy and criteria.

www.webofknowledge.com, www.scopus.com). The keywords were "tendon detraining", "tendon detraining associated damage", "tendon sudden detraining", "tendon disuse", "tendon discontinuous training associated damage", "tendinopathies and discontinuous training", "tendinopathies and detraining", "tenocytes detraining associated damage", "tenocytes discontinuous training associated damage". We sought to identify studies in which tendon detraining was examined. Publications from 2005 to 2015 (original articles in English) were included. The reference lists from the articles included in this review were analyzed to recognize additional studies that were not found by the initial search. A public reference manager (www.mendeley.com) was used to delete duplicate articles.

RESULTS

An initial literature search yielded 134 references. Fifty-three articles were identified using www.pubmed.org, 70 articles using www.webofknowledge.com and 11 articles were found in www.scopus.com. The resulting references were submitted to a public reference manager (Mendeley 1.13.8, www.mendeley.com) to delete duplicate articles. Of the 76 remaining articles, 15 publications were extracted based on the title for further analysis. Abstracts and whole articles were then reviewed to ascertain whether the publication met the inclusion criteria and eight articles (four preclinical studies, one *in vitro* and three *in vivo*, and four clinical studies) were considered appropriate for the review (**Figure 1**). From the reference lists of the included articles, no supplementary publications were identified. We did not perform meta-analyses of the selected studies, but quoted the results in a descriptive fashion.

Preclinical Studies

This revised literature comprised four preclinical studies, an *in vitro* and three *in vivo* reports, respectively on tenocytes from patellar tendon (Salamanna et al., 2015) and on patellar (Frizziero et al., 2011, 2015) and gastrocnemius (Foutz et al., 2007) tendon of detrained animals (**Table 1**). Concerning the *in vitro* study patellar tendon tenocytes from rats subjected to training and to sudden detraining were examined. Rats were trained for 10 weeks on a treadmill (speed of about 25 m/min, corresponding to ∼65–70% VO_2max) and successively caged without exercise for further 4 weeks. Tenocytes from patellar tendon were cultured to evaluate morphology, viability, proliferation and metabolic activity. It was found that detraining in the short-term alters tenocyte synthetic and metabolic activity (C-terminal-propeptide of type I collagen, collagen III, fibronectin, aggrecan, tenascin-c, interleukin-1β, matrix-metalloproteinase-1 and -3). These results indicated that tenocytes do not merely have a passive role but play an important function during detraining (Salamanna et al., 2015). Similarly results were found by the same authors also when the patellar tendons of detrained rats were studied by histology and histomorphometry (Frizziero et al., 2011, 2015). In fact, the studies showed alteration in tendon morphology and also in its enthesis due to discontinuation of training. These alteration involved proteoglycan content, collagen fiber organization with an increase of collagen III and a decrease of collagen I, which means less resistance to stress, and a related increased risk of rupture. Differently from the above mentioned studies, Foutz et al. (2007) investigated the mechanical adaptability responses due to disuse on the biomechanical properties of the gastrocnemius tendon of chicks. Chicks were trained for 3 weeks on a treadmill (speed of 0.22 m/s, for 5 min) and successively immobilized in a whole body suspension system for further 2 weeks. It was found that structural strength and toughness of the gastrocnemius tendon were reduced by 10 and 30%, respectively, whereas the material strength, material toughness, and material stiffness of the tendon increased by approximately 75, 65, and 70%, respectively. These results showed that the chicken gastrocnemius tendon reacts to mechanical disuse as foretold by the mechanobiology process (Foutz et al., 2007).

TABLE 1 | Preclinical studies on role of detraining in tendon mechanobiology.

Experimental set-up	Type of tendon	Control group	Training protocol	Detraining protocol	Analysis	Main results	Reference
In vitro model	Rat patellar tendon tenocyte	Untrained patellar tendon tenocyte Trained patellar tendon tenocyte	10 week on a treadmill (∼65–70% VO_2max)	Caged without exercise for 4 weeks	Transmission- electronic-microscopy, C-terminal-propeptide of type I collagen, collagen III, fibronectin, aggrecan, tenascin-c, interleukin-1β, matrix-metalloproteinase-1 and-3	Altered tenocyte synthetic and metabolic activity	Salamanna et al. (2015)
In vivo model	Chicken gastrocnemius tendon	No control group	3 week on a treadmill (speed of 0.22 m/s, for 5 min)	Controls or immobilized for 2 weeks	Tendon midregion cross-sectional area and biomechanical properties	Gastrocnemius tendon responds to mechanical disuse as predicted by the mechanobiology process	Foutz et al. (2007)
In vivo model	Rat patellar tendon	Untrained patellar tendon Trained patellar tendon	10 week on a treadmill (∼60% VO_2max)	Caged without exercise for 4 weeks	Collagen fiber organization and proteoglycan content	Low proteoglycan content and collagen fiber organization	Frizziero et al. (2011)
In vivo model	Rat patellar tendon	Untrained patellar tendon Trained patellar tendon	10 week on a treadmill (∼65–70% VO_2max)	Caged without exercise for 4 weeks	Structure and morphology (modified Movin score, tear density, collagen type I and III)	Altered structure and morphology with the highest Movin score values, the highest percentage of collagen III and the lowest of collagen I	Frizziero et al. (2015)

Clinical Studies

The PubMed, Web of Knowledge and Scopus search strategy identified four clinical papers that examined the impact that detraining may have on tendons (**Table 2**). Several studies showed that tendon characteristics influence the performances during stretch-shortening cycle exercises (Bojsen-Møller et al., 2005; Kubo et al., 2007; Stafilidis and Arampatzis, 2007); thus, information on the time course of changes in tendon characteristics during training and detraining is critical for the progress of performances in the athletic field. To evaluate the time course of modifications in mechanical and morphological properties of tendon during detraining, Kubo et al. (2010) examined these variables in eight volunteered men that executed unilateral knee extension exercise in a seated position. Subjects were trained 4 times per weeks for 3 months and detrained for the following 3 months. Results of this study showed that tendon stiffness was significantly increased after 3 months of training, while the maximal elongation was unaltered. Conversely, during the detraining period, tendon showed greater values of maximal elongation compared to the post-training, and tendon stiffness decreased to the pre-training levels after 2 months of detraining (Kubo et al., 2010). With a similar methodology, the same authors in 2012 focused more specifically on the alterations found in the human Achilles tendon during training and detraining (Kubo et al., 2012). In addition, they measured the blood volume and oxygen saturation of tendon, and evaluated the serum concentrations of markers of collagen type I synthesis. Results were similar to the previous study ones: the elongation values did not change after training but increased significantly during detraining; tendon stiffness increased only after 3 months of training and rapidly decreased during detraining. Thus, authors showed that during detraining, the sudden decrease in tendon stiffness might be linked to modifications in the structure of collagen fibers within the tendon. In addition, no significant alterations in blood supply or collagen synthesis were observed (excluding an increase in procollagen peptides after 2 months of training; Kubo et al., 2012).

Recently McMahon et al. (2013) evaluated the patella tendon properties during detraining (1 month), after a 3 months period of training with different strains. The patella moment arm, the perpendicular distance between the tibiofemoral contact point and the mid-portion of the tendon, was estimated using dual-energy x-ray absorptiometry (DEXA) scan images. Tendon elongation and stiffness were measured by ultrasonic analyses and tendon forces were calculated as the ratio between the measured torque and the patella moment arm. Furthermore, they evaluated the circulating transforming growth factor (TGF)-β1 levels as it is associated to exercise-induced response to mechanical loading of muscle and tendon. The authors found no significant alterations in patella tendon dimensions or circulating TGF-β1 levels following training or detraining. However, the training groups with the muscle-tendon complex at a lengthened position or over a wide range of motion better maintained adaptations compared to the training in a shortened position subsequent to detraining, with a pattern of slower loss of progress at the early phase of detraining in all training groups.

TABLE 2 | Clinical studies on role of detraining in tendon mechanobiology.

Type of tendon	Patients	Training protocol	Detraining protocol	Analysis	Main results	Reference
Patellar tendon	8 (training group); 6 (control group)	Unilateral isometric knee extension, 4 times/week, 3 months	Return to usual levels of physical activity, 3 months	- Tendon elongation by ultrasounds; - Cross-sectional areaby MRI	Greater values of tendon elongation, decrease in tendon stiffness during detraining	Kubo et al. (2010)
Achilles tendon	9 (training group); 7 (control group)	Unilateral (left side) isometric plantar flexion exercise, 4 times/week, 3 months	Return to usual levels of physical activity, 3 months	- Tendon elongation by ultrasounds; - Cross-sectional areaby MRI; - Blood supply and oxygen saturation; - Serum concentration of BAP and P1P by ELISA	Tendon elongation increased and stiffness rapidly decreased after detraining	Kubo et al. (2012)
Patellar tendon	10 (training with the MTC at a shortened position); 11 (MTC at a lengthened position); 11 (wide range of motion); 10 (control group)	Resistance training, 3 times/week 8 weeks	4 weeks of detraining	- Patella moment arm by DEXA; - Tendon elongation and stiffness by ultrasounds; - Circulating TGF-β1 levels by ELISA	No significant alterations in patella tendon dimensions or circulating TGF-β1 levels following training or detraining in any of the groups	McMahon et al. (2013)
Achilles tendon	10 (training on inclined ground); 10 (training on plain ground)	Plyometric training	4 weeks of detraining	Aponeurosis strain of MG	Strain was decreased from 22.7% (±0.05) to 16.3% (±0.05) after detraining period	Kannas et al. (2015)

Finally Kannas et al. (2015) analyzed the effect of 4 weeks of detraining on the mechanical properties of medial gastrocnemius aponeurosis into two groups that performed plyometric training on incline and plane ground. They evaluated the aponeurosis strain of medial gastrocnemius and found that it decreased after detraining; the ankle muscle tendon complex properties withdrew to the pre-training values with lower performances. These findings suggested that after 4 weeks of detraining, ankle muscle tendon complex properties withdraw to the pre-training values with lower performance (Kannas et al., 2015).

DISCUSSION

The tendon is a connective tissue responsible for the transmission of force from the muscular tissue to the bones, promoting body movement. It is not a static tissue, preferentially it adapts itself in compliance to the level, direction and frequency of the load that is applied to it with a process of remodeling possibly executed by tenocytes.

It was shown that appropriate mechanical loads are useful to tendons by improving their anabolic processes and it is undertaken or prescribed for different reasons such as sports performance, general health, functional maintenance, recovery (e.g., following injury, illness/diseased states) and also to compensate the effects of ageing. However, extreme mechanical loads are harmful to tendons by bringing catabolic processes such as matrix degradation. Immobilization or disuse of tendons also leads catabolic effects on it. Differently there are few data that examined the impact that detraining may have on tendons. Thus, the present descriptive systematic literature review tried to summarize the effects of discontinuing physical activity on tenocyte metabolism and/or in tendon morphology in order to elucidate the mechanism behind these changes.

All examined studies, both preclinical and clinical, observed that discontinuing activity negatively influence tendon structure and morphology, albeit with differences in the training and/or detraining protocols, in the types of tendons, in subjects involved, in the study design or in the experimental setting involved. The results of all these studies suggested that after a period of sudden detraining (such as after an injury) physical activity should be restarted with caution and with appropriate rehabilitation programs because cessation of activity causes modifications in tenocytes and tendons metabolism, morphology, i.e., in collagen type I and III synthesis, collagen organization, cellularity, vascularity, proteoglycan content, tear density, mechanical properties.

Notwithstanding the alterations highlighted in the reviewed articles after tendon detraining, some limitations of the examined studies should be also considered. In fact, this systematic review has as its main focus not only to bring together major works involving major changes in morphological and structural properties of tendons during detraining, but also to examine the methodological process on which the articles were based to assess the trustworthiness of the results found.

In relation to the results obtained in the *in vitro* study examined in this review (Salamanna et al., 2015), that showed a decrease of tendon mitochondrial area, rough endoplasmic reticulum area, C-terminal propeptide of type I collagen, fibronectin, aggrecan and tenascin-c synthesis and presence of inflammatory cytokine production, we have to consider that tenocytes from animals subjected to sudden detraining were studied. In addition, results were obtained in *in vitro* cultured cells, which were not any longer structured into tissues, but in monolayer and static conditions. Thus, it is probable that the performance of explanted tendon cells is not equal to the performance of tendon cells in their native matrix environment *in vivo* (Fu et al., 2008; Leigh et al., 2008). However, these results indicated that the tendon does not operate as a inert connector between muscles and bone, but dynamically responds to mechanical loading.

The three preclinical studies examined in this review employed a rat or chicken animal model that may not be fully representative of human conditions but the invasive analyses conducted in these studies permitted a depth investigation for the advancement of knowledge of many aspects on tendon response to detraining (Foutz et al., 2007; Frizziero et al., 2011, 2015). Moreover, looking at the literature, rat and rodents are the most used animals when mechanical load with treadmill running is used (Warden, 2009; Lui et al., 2011). In fact, the results of these *in vivo* studies demonstrated that the adopted running protocol did not induce tendinopathy or other pathologic changes in hindlimbs. Another methodological process that must be considered is that in these studies all morphometric parameters were measured by 2D image analysis, while other investigation methods, such as micro-MRI, may allow a more in-depth understanding of tendon structure. However, as for the reviewed *in vitro* paper, these *in vivo* results provide interesting data for both sports medicine practitioners and orthopedic surgeons, wishing to prevent the pathological or degenerative modification that affect these structures.

Great variability was noted in the four clinical studies (Kubo et al., 2010, 2012; McMahon et al., 2013; Kannas et al., 2015) that analyzed the effects of detraining. In fact, these studies involved different tendons (Achilles, gastrocnemius, patellar), different types of exercise (isometric knee extention, resistance training, plyometric training on incline and plane ground), different training and detraining periods (3 and 4 months) and different types of analyses (Dual Energy X-Ray Absorptiometry, ultrasonography, electromyography). Furthermore, it is important to point out that the different effects of detraining on tendons depends not only on the above mentioned variables, but also on the patient intrinsic characteristics, that are affected by age, gender, drug assumption, the presence of systemic or genetic or endocrine diseases (i.e., obesity, diabetes, Cushing syndrome, hypercholesterolemia, osteoporosis). In fact, recently it was shown that proliferation and synthetic activity of tenocytes are negatively affected by aging and estrogen deficiency (Torricelli et al., 2013). In addition, clinical studies did not permit a depth understanding of the alteration in tendon metabolism and morphology (i.e., expression of type I collagen, fibronectin, aggrecan and tenascin-c synthesis and/or presence of inflammatory cytokine, cellularity, vascularization, fibers arrangements etc.). However, despite these limitations these clinical studies indicate that tendons may be susceptible

to detraining. These findings could have a direct relevance to functional rehabilitation practices showing that after a period of sudden detraining, physical activity should be restarted with caution.

Despite the fact that the examined studies showed a potential negative effect of detraining on tenocytes and tendons, there is a paucity of preclinical and clinical studies that examined the importance that cessation of training may have on tendon. These results should be confirmed by other preclinical and clinical research in order to completely comprehend the effect of detraining on tendons. In particular, several aspects should be further studied and refined in order to improve our understanding on the role of detraining in tenocytes and tendon mechanobiology: (1) standardization of the training and detraining protocols in both preclinical and clinical research; (2) development of systems that reproduce tendon detraining in culture with high reliability to native tendon; (3) comprehend how tenocytes respond to detraining and how they mechano-regulate their response; (4) evaluate the presence of altered tendon structure and/or morphology due to detraining in its various stages; and (5) evaluation of the role of other tissues (bone, muscle, nerve, vascularity, etc.) on tendon mechanobiology during detraining. Finally an integrated, collaborative multi-disciplinary multiscale approach is likely to yield the greatest advances in this field.

AUTHOR CONTRIBUTIONS

AF: has conceived the study and was involved in drafting the manuscript, FS: has conceived the study and was involved in drafting the manuscript, EDB: was involved in the literature search and in the data analysis, FV: was involved in the literature search, GG: was involved in drafting the manuscript, NNA: was involved in the literature search, SM: participated in drafting the manuscript and in the data analysis, MF: has conceived the study and was involved in drafting the manuscript.

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Fibrillins in Tendon

Betti Giusti[1,2] *and Guglielmina Pepe*[1,2]*

[1] Department of Experimental and Clinical Medicine, Excellence Centre for Research, Transfer and High Education for the Development of De Novo Therapies (DENOTHE), University of Florence, Florence, Italy, [2] Marfan Syndrome and Related Disorders Regional (Tuscany) Referral Center, Careggi Hospital, Florence, Italy

Tendons among connective tissue, mainly collagen, contain also elastic fibers (EF) made of fibrillin 1, fibrillin 2 and elastin that are broadly distributed in tendons and represent 1–2% of the dried mass of the tendon. Only in the last years, studies on structure and function of EF in tendons have been performed. Aim of this review is to revise data on the organization of EF in tendons, in particular fibrillin structure and function, and on the clinical manifestations associated to alterations of EF in tendons. Indeed, microfibrils may contribute to tendon mechanics; therefore, their alterations may cause joint hypermobility and contractures which have been found to be clinical features in patients with Marfan syndrome (MFS) and Beals syndrome. The two diseases are caused by mutations in genes FBN1 and FBN2 encoding fibrillin 1 and fibrillin 2, respectively.

Keywords: tendon, fibrillin, elastic fibers, oxytalan fibers, contractures, Marfan syndrome, extracellular matrix

INTRODUCTION

Tendon is a complex hierarchical structure mainly made of collagen fibrils, in which extracellular matrix (ECM) is build up by tenocytes. More fibrils, mainly made of collagen-type-I, make fibers which are organized in fascicles which are embedded by the endotenon sheath and all together form the final tendon structure (**Figure 1**).

While collagen fibrils' structure and mechanism have been widely studied, the remaining ECM proteins, in particular fibrillins, in human tendons have been poorly investigated. Few data are available on fibrillins in tendons. Elastic fibers (EF) are widely distributed among tendons. They are made of elastin which is the core and has the capacity to recover completely from total deformation (Kannus, 2000). It has been hypothesized that insoluble elastin provides tendon with elastic recoil and resilience (Butler et al., 1978), as reported in blood vessels and skin (Kielty et al., 2002). Microfibrils, mostly made of fibrillin-1 and fibrillin-2, constitute a scaffold around elastin, they colocalize in tendon, mostly together with elastin (Mithieux and Weiss, 2005). Tropoelastin, during elastogenesis, is deposited on microfibrils and stabilized by cross-links made up by lysyl oxidase (Kielty, 2006). EF, are made of oxytalan fibers (OF, mostly fibrillin-1 and fibrillin-2 microfibrils) and elastin in most tissues comprised tendons and ligaments, as shown in dogs (Ritty et al., 2002; Smith et al., 2011). In canine tendon (flexor digitorum profundis) OF are present with different distribution of the two fibrillins in different places, with or without elastin (Ritty et al., 2002, 2003a,b). It is known that EF organization may vary with age (Kannus, 2000).

The joint movement is made possible by the force created by the muscle and transmitted to the bone through the tendons. Tendons are exposed to transversal, rotational, longitudinal forces, pressures and contusions. Its internal structure protects from these forces (Józsa and Kannus, 1997). Tendon tissue contains adhesive glycoproteins, among several non-collagenous

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***Correspondence:**
Guglielmina Pepe
guglielmina.pepe@unifi.it

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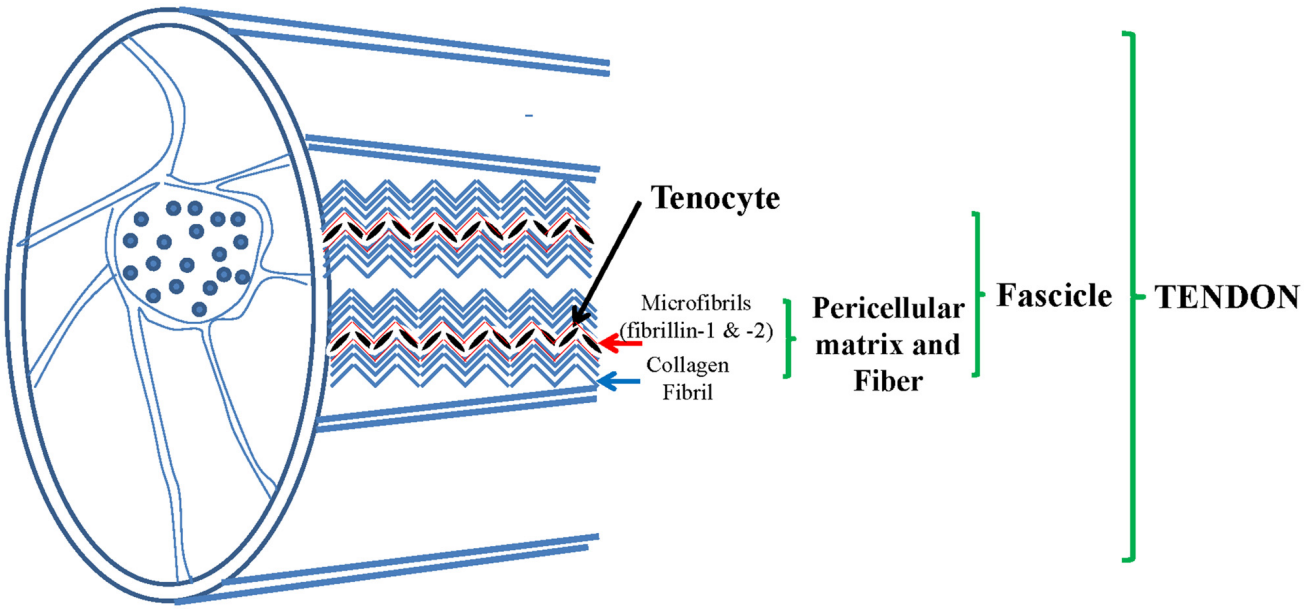

FIGURE 1 | Tendon, a complex structure made of collagen fibers constituted by collagen type I and pericellular matrix (PCM) mainly made of elastic fibers (EF): fibrillin 1 and 2, elastin, collagen type VI and others. Adapted from Grant et al. (2013).

proteins, which bind other macromolecules or cell surfaces together (Kannus et al., 1998). Fibronectin, undulin, tenascin-C and thrombospondin (Miller and McDevitt, 1988; Józsa et al., 1991; Kannus et al., 1998) were detected among the tendon belly. Vascular walls of the tendons display the presence of laminin (Józsa et al., 1991) which is highly localized at the myotendinous junction (Järvinen et al., 1991; Kvist et al., 1991).

Ninety-five percent of tendon cells are tenoblasts (in youngs) and tenocytes (in adults); the remaining 5% are chondrocytes localized at the pressure and insertion sites, synovial cells on the tendon surface of the tendon sheath, and vascular cells (smooth muscle cells of the arterioles capillary, endothelial cells) in the endotenon. In diseases, myofibroblasts, inflammatory cells, macrophages, may be revealed in tendons (Józsa and Kannus, 1997). Structure and diameters of tendons vary greatly in size and at different age (de Campos Vidal and de Carvalho, 1990). Decrease in muscle-mass and strength, as well as alteration of tendon and bone structure are observed during aging (Keller and Engelhardt, 2014). These alterations are mainly due to collagen synthesis decrease, free radicals expression increase and metabolism imbalance in favor of catabolic activity (Tsai et al., 2011; Yu et al., 2013).

ANIMAL MODEL DATA

Animal models have been used to characterize the structure of tendons. Only very recently, some human tendons' structures have been analyzed.

Bovine Model

Analyses of deep digital flexor bovine's tendons with no sign of tissue damage (young adult steers, age 18–24 months) shows that EF (elastin appears broadly distributed in tendon) are particularly localized around tenocytes and between fascicles. Their close localization suggests that EF, being part of the pericellular matrix (PCM), therefore forming a network with tenocytes, may influence cellular function. Beside, the network enriched by EF allows tenocytes to exert mechano-biological responses to load. EF, soon after the removal of load, support tenocytes in going back to their previous physiological configuration (Screen et al., 2004). ECM plays an important rule in keeping tissue homeostasis; its disruption may cause a spectrum of disorders (Ingber, 2003). Being strictly associated to cells, EF may participate to cell attachment. Fibrillin-1 binds to integrins and collagen-type-VI (Midwood and Schwarzbauer, 2002) which is an important component of PCM in tendon (Carvalho et al., 2006). OF link cell to collagen-type-VI, which binds to fibrillar collagens. The interaction and integration of EF with the surrounding matrix is facilitated by colocalization of fibrillin-1 and perlecan in many connective tissues including the anterior cruciate ligament (Hayes et al., 2011). OF not only withstand mechanical deformations but also regulate transforming growth factor beta (TGF-β) bioavailability (Vehviläinen et al., 2009) since, bound to fibrillin-1, it is protected from metalloproteinases activity while, when fibrillin-1 is altered, it may be released and exert a tissue remodeling process. According to the mechanism of elastogenesis in which tropoelastin deposition is performed on a microfibrils' template, elastin in tendon

colocalizes with fibrillin-1 (Fahrenbach et al., 1966). OF are present in bovine tendon, as well as cruciate ligament (Smith et al., 2011) and flexor digitorum profundus (FDP) tendon (Ritty et al., 2002) in dog tendon. Although microfibrils are stiffer and less extensible than elastin (Sherratt et al., 2003) its capacity to recover from deformations confirms a possible mechanical role similar to that of EF (Baldock et al., 2001).

Dog Model

EF, OF and microfibril-associated glycoproteins (MAGPs) 1 and 2 distribution in the FDP tendon of dogs, was studied and characterized by Ritty et al. (2002). The fibrocartilaginous, avascular/tensional and insertion, three functionally distinct regions of the FDP tendon were investigated by immunohistochemical analysis for the five above mentioned proteins. Both biochemical and histochemical analysis of desmosine content, an elastin-specific cross-link, detected elastin in all regions. Fibrillins were found not only with elastin but also alone around internal fibroblasts. Although colocalized, fibrillin-2 was more abundant inside the tendon while fibrillin-1 was more present in outer cell layers. MAGP-1 and MAGP-2 were highly present near the tendon insertion to bone but also distributed along the tendon (Ritty et al., 2002). In adult dogs with no evidence of knee osteoarthritis, Smith et al. (2011) demonstrated that OF and EF were widespread in both cruciate ligaments, in particular in ligament fascicles, parallel to collagen bundles. Abundant fibrillin-1 and fibrillin-2 reach OF, were observed. Distribution of EF indicated a possible mechanical role in bundle reorganization following ligament deformation. Presence and location of fibrillin-2 OF in ligament differs from the solely fibrillin-1-containing OF previously described in tendon suggesting differences between ligament and tendon (Smith et al., 2011). These data obtained in dogs suggested that OF may contribute to tendon mechanics, as joint hypermobility and contractures found in patients with Marfan syndrome (MFS, OMIM 154700) and Beals syndrome (OMIM 121050), caused by mutations in the genes encoding fibrillin-1 and fibrillin-2, respectively (Urbán and Boyd, 2000; Gupta et al., 2002, 2004). At present, no data are available on tissular and molecular mechanisms underlying the manner that both syndromes affect the body's joints. Moreover, no data are reported in literature regarding joint hypermobility and contractures in other fibrillinopathies (ectopia lentis, Shprintzen–Goldberg syndrome, Weill–Marchesani syndrome, familial or isolated aortic aneurysms) caused by alteration of fibrillin-1 and fibrillin-2.

Mouse Model

Both collagen and intermolecular collagen cross-links hydroxylysyl and lysylpyridinoline content of flexor digitorum longus tendons were analyzed in *Fbn2* gene null mice. Results showed decreased collagen cross-links when compared to wild type mice. Thus, loss of fibrillin-2, may result at the end in dysregulation of lysyl oxidase activating enzymes and may provide a mechanistic explanation for the reduced level of lysyl oxidase catalyzed collagen cross-links in the *Fbn2* null mice tendon. *Fbn2* null mice bone morphology, investigated through micro computed tomography, displays a focal area of decreased bone length in the extremities as compared to wild type mice (Boregowda et al., 2008). Another phenotypic trait is a "fusion" of some elements of the third and fourth digits (phalanges and metacarpals; Arteaga-Solis et al., 2001). Work from other researchers suggests that fused digits are due to a failure of interdigital cell apoptosis and that the failure to septate depends, at least in part, on dysregulation of bone morphogenetic proteins (BMPs; Dahn and Fallon, 2000). During hand development decreased bone growth, detected in the *Fbn2* null mice, defines a role for the *Fbn2 gene*. TGF-β superfamily members are known to be implicated in limb and skeleton formation (Arteaga-Solis et al., 2001; Bandyopadhyay et al., 2006); in the absence of fibrillin-2 protein, dysregulation of growth factors may cause morphological alterations. Beside, the skeletal phenotype of *Fbn1* hypomorphic mice, where long bone overgrowth was observed (Pereira et al., 1999), is the opposite of the short skeletal phenotype of the *Fbn2* null mice. Pereira et al. (1999) demonstrated that homozygous *Fbn1* hypomorphic mice (mgR) produce about 25% of *Fbn1* normal amount. mgR animals present in the skeletal and aortic manifestations which mimic those of MFS patients. Such mouse model also manifests severe kyphosis, proposed to be due microfibril-rich ligaments' and tendons' loss of tensile strength (Zhang et al., 1995). Mechanisms of bone overgrowth in MFS have been longly discussed. In the skeleton, gain-of-function mechanism of Fbn1 mutations has been suggested, while loss-of-function mechanism was indicated in cardiovascular and ocular systems (Dietz et al., 1994). Further evidence exists suggesting that microfibrils exert negative control on bone growth for their double role in preserving periosteal tension and tensile strength of ligaments and tendons (Zhang et al., 1995; Keene et al., 1997). Homozygous Fbn1 hypomorphic mice skeletal data support the involvement of tendons in some fibrillinopathies, as shown in MFS by Melchiorre et al. (2016). Indeed, Zhang et al. (1995) showed that Fbn1 and Fbn2 genes are differentially expressed during developmental stages and various tissues and suggested that Fbn2 regulates the early EF assembly and Fbn1 plays a prevalent role in providing structural support.

HUMAN TENDON DATA

In human tendons, collagen bundles are made of collagens types-I and -III. Fibril diameter may be regulated by collagen-type-III. Type III bundles may participate to the attachment of the periosteum or of tendons and ligaments. The distribution of fibrillin parallels that of collagen-type-III suggesting that OF are positioned within and around collagen-type-III fibers in human tendon bundles (Keene et al., 1991).

Supraspinatus tendons, analyzed for the first time in humans, show that collagen-type-VI is localized strictly together with elastin and fibrillin-1 in the PCM region of supraspinatus tendon, as confirmed by animal model studies (Thakkar et al., 2014).

Collagen-type-VI provides tendon's structural integrity and, due to its cell–matrix and matrix–matrix interactions, functions

as a key regulator of matrix signals (Bonaldo et al., 1990; Kuo et al., 1997). In human aortic media, it has been reported that fibrillin-1 and collagen-type-VI may form bundles. These data are of interest in relation to inherited connective tissue disorders because it suggests that a mutation in one of the two proteins affects the bundles (Dingemans et al., 2000).

EF display three major functions: provide mechanical properties, including elastic recoil and resilience to tissue (Butler et al., 1978), lead the activity of the TGF-β family (Charbonneau et al., 2004; Feng and Derynck, 2005) and participate to handle cell migration, survival and differentiation (Ito et al., 1997). Fibrillins exert the structural role through the temporal and hierarchical assembly of EF. On the other hand, fibrillins play the instructive role by their ability of sequestering TGF-β and BMP complexes in the ECM. It is well known that fibrillin mutations in humans and animal models determine TGF-β signaling perturbation (Ramirez and Sakai, 2010).

EF localize around tenocytes and between collagen fascicles participating to the structure protection during extended periods of loading (Ritty et al., 2002). The knowledge of the interaction between EF and TGF-β signaling confirms the connection demonstrated in humans through mutations in FBN1, TGFBR1 and TGFBR2 genes that are known to cause MFS and overlapping disorder as Loeys-Dietz syndrome (LDS), familiar thoracic aorta aneurysms and dissections (Giusti et al., 2016). It is known that fibrillin-1 interacts with TGF-β through a protein complex (Pepe et al., 2016).

Moreover, tenocytes distributed along tendon PCM join to it forming an array. The array structure replies as a unit to biomechanical and biochemical signals. PCM mechanical properties were already found altered in other tissues. Of particular significance are mechano-biological mechanisms, which have been shown to be significantly altered by the mechanical properties of PCM in other tissues (Wang, 2006; Eyckmans et al., 2011). Concerning heart function and structure, it was observed that in addition to furnish tensile strength and elasticity to tissues, fibrillin-1 assemblies also regulates cell behavior by interacting with integrin receptors and by modulating latent TGF-β bioavailability (Ramirez and Rifkin, 2009; Ramirez and Sakai, 2010).

Tendon tears' formation, causing damage to structure and to mechanics of cell microenvironment, may represent the clue for understanding pathologic modification and regeneration of tissue (Thakkar et al., 2014). Collagen-type-VI and fibrillin-1 were more abundant than the widely distributed elastin, as confirmed by qualitative images of large tissue tears from torn supraspinatus tendon showing an extended disruption of collagen-type-VI microfibrils. Beside, the presence of increased collagen-type-III is a biomarker of great potential for healing (Thakkar et al., 2014). Studies from other groups are required to confirm the mechanisms causing tendon degeneration and its relation to rotator cuff disease prognosis. To explain, it is important to outline the tight interaction between collagens and EF while in humans a mutation in one of these genes-proteins display wide phenotypical heterogeneity also inside the same family. It is reasonable to hypothesize that the damage is extended to other proteins interacting with the mutated protein.

ELASTIC FIBERS AND PATHOLOGIES WITH TENDON INVOLVEMENT

During EF formation in late prenatal and neonatal development, OF constitute a three-dimensional scaffold for the assembly of elastin (Urbán and Boyd, 2000). Insoluble elastin provides to EF the property of elastic recoil. In addition to the mechanical properties of resilience, EF undergo very little turnover in normal adult tissues, with the exception of the uterus. In adult tissue, new EF synthesis causes accumulation of dysfunctional EF present in common disorders such as emphysema, hypertension, actinic elastosis (abnormal elastin accumulation) and aortic aneurysms (Kielty, 2006).

Heritable, monogenic diseases of EF are represented by fibrillinopathies, elastinopathies and, more recently, TGF-β-pathies (Bradley et al., 2016).

For long time congenital fingers contractures have been considered one of the cardinal manifestations of congenital contractural arachnodactily (CCA) and associated to muscle alterations and to FBN2 mutations, the gene encoding fibrillin-2. Recently, we suggested the involvement of tendons in congenital fingers' and toes' contractures observed in Marfan patients, known to display only elbows contractures (Loeys et al., 2010), on the basis of ultrasound analysis. A reduction of thickness of all fingers' and toes' tendons was detected, suggesting an association between these findings and structural modifications in connective tissue (Melchiorre et al., 2016). The pilot study was performed on 13 Marfan patients diagnosed in our Center. Since fingers and toes contractures were reported for the first time in MFS (Melchiorre et al., 2016), patients which are known to display elbows contractures (Loeys et al., 2010), we searched for such manifestations in 100 Marfan patients consecutively coming at our Center for routinary controls. Toes' contractures and fingers' contractures were present in 30% and 12% of patients, respectively. Reduced elbow contractures were found in 37% of patients (Melchiorre et al., 2016). These data suggest the opportunity of performing histological analysis of contractured tendons in Marfan patients to verify the structural alteration of microfibrils and a revision of the tendon contractures (localization, expression) present in heritable connective tissue disorders, since contractures are present also in other diseases such as LDS, Ehlers-Danlos syndromes and X-linked cutis laxa. We do not know if and in which of the above mentioned disorders tendons are involved and if they are present but never reported in other inherited connective tissue disorders.

Fibrillinopathies: MFS, Neonatal MFS, CCA

MFS. Mutations in *FBN1*, a gene located on chromosome 15q21.1 that encodes fibrillin-1, result in the ocular, cardiovascular, osteoarticular (among these: elbows' contractures; **Table 1**), pulmonary, skin and central nervous features characteristic of MFS (Loeys et al., 2010; Giusti et al., 2016; Pepe et al., 2016).

FBN1 mutations also cause a group of disorders called fibrillinopathies type 1 which include ectopia lentis,

TABLE 1 | Tendons' contractures, contractures and inflammation in heritable connective tissue disorders.

Connective tissue disorder/Gene	Elbows	Wrist	Hand digits	Hips	Knees	Feet toes
MFS/*FBN1*	+	?	+	?	?	+
nMFS/*FBN1*	+	+	?	+	+	?
CCA/*FBN2*	+	+	+	+	+	+
LDS1/*TGFBR1*	?	?	+	?	?	+
LDS2/*TGFBR2*	?	?	+	?	?	+
LDS3/*SMAD3*	?	?	+	?	?	?
LDS4/*TGFB2*	?	?	?	?	?	+
LDS5/*TGFB3*	?	?	?	?	?	+
EDS/*COLs*	Ligamens' and tendons' rupture, short Achilles tendon, tendon stress/over use, damage/rupture, tenosynovitis, tendinitis, bursitis					

MFS, Marfan Syndrome; FBN1, fibrillin 1; nMFS, neonatal Marfan Syndrome; CCA, Congenital Contractural Arachnodactyly; FBN2, fibrillin 2; LDS1, Loeys-Dietz Syndrome 1; TGFBR1, Transforming Growth Factor Beta Receptor 1; LDS2, Loeys-Dietz Syndrome 2; TGFBR2, Transforming Growth Factor Beta Receptor 2; LDS3, Loeys-Dietz Syndrome 3; SMAD3, Mothers Against Decapentaplegic, Drosophila, Homolog of, 3; LDS4, Loeys-Dietz Syndrome 4; TGFB2, Transforming Growth Factor Beta 2; LDS5, Loeys-Dietz Syndrome 5; TGFB3, Transforming Growth Factor Beta 3; EDS, Ehlers-Danlos Syndrome; COLs, Collagens. ? = unknown; + = reported.

Weill-Marchesani syndrome, familial ascending aortic aneurysms and dissections, Shprintzen-Golden syndrome, MASS phenotype, kyphoscoliosis, isolated skeletal features, familial arachnodactyly, neonatal MFS (nMFS), the most severe phenotype of MFS. In this last disease, *FBN1* mutations mainly positioned between the central exons 24–32, displays the following clinical features: arachnodactyly, campodactyly (congenital contractures of elbow, wrists, digits and toes; **Table 1**), micrognathia, crumpled ears, rocker bottom feets (arachnodactytly, overlapping toes and hypoplasia of calf muscles), loose redundant skin creating a senile look of the facies, severe cardiac valve insufficiency and aortic dilatation (Buntinx et al., 1991).

A related disease, CCA (OMIM 121050), was shown associated to mutations in *FBN2*, a second fibrillin gene on chromosome 5q23.3. CCA is clinically characterized by multiple flexion contractures (elbows, knees, hips, wrists, fingers and toes; **Table 1**), arachnodactyly, severe kyphoscoliosis, abnormal pinnae and muscular hypoplasia (Putnam et al., 1995; Gupta et al., 2002). This work on disease-associations also contributed to the realization that fibrillin-1 and fibrillin-2 are major components of elastic microfibrils. The presence of multiple flexion contractures in both nMFS and CCA has never been investigated in terms of tendon involvement.

Recently, *FBN2* variants such as *FBN2* rs331079 have been recognized as predisposing factors for Achilles tendinopathy (AT; Khoury et al., 2015). Other gene variants have already been found as predisposing factors for AT, among these are variants within Collagen, type V, alpha 1 (*COL5A1*; Mokone et al., 2006), matrix metallopeptidase 3 (*MMP3*; Raleigh et al., 2009), TIMP Metallopeptidase Inhibitor 2 (*TIMP2*; El Khoury et al., 2013), Tenascin C (*TNC*; Collins and Raleigh, 2009), growth differentiation factor 5 (*GDF5*; Posthumus et al., 2010) genes. Mutations in elastin encoded by ELA gene (ch7q11) are associated to supravalvular aortic stenosis and Williams-Beuren syndrome characterized by narrowing of whole arterial three and increased elastinolytic activity and autosomal and recessive cutis laxa with redundant, loose and inelastic skin, pulmonary emphysema and aortic disease. The milder dominant form displays also genital prolapse, diverticula, hernias, pulmonary artery stenosis (Kielty, 2006).

TGF-β-Pathies. Loeys-Dietz Syndrome (LDS Types 1–5)

Since more than 10 years, MFS has been associated with increased TGF-β signaling (Neptune et al., 2003). Thus the molecular mechanism underlying MFS is more complex than a single dominant mutation in FBN1, which is due to a perturbation of TGF-β signaling. Fibrillin-1 participates to the correct activation of TGF-β since it is part of the large protein complex which keeps TGF-β inactive until TGF-β links to its receptors TGFBR1 and 2 (Gelb, 2006).

LDS (OMIM 609192) is an autosomal dominant disorder of connective tissue caused by heterozygous mutations in genes codifying for TGFB receptor 1 or 2 (TGFBR1, LDS1 or TGFBR2, LDS2). Its cardinal manifestations are: hypertelorism, cleft palate or bifid uvula, arterial tortuosity and/or arterial/aortic aneurysm. LDS displays four major clinical manifestations: vascular ectasias and tortuosities, skeletal features, facial dismorphology and skin manifestations. Among skeletal features, contractures of feet (talipes equinovarus) and fingers (campodactyly; **Table 1**) are common features (Loeys et al., 2005, 2006).

Mutations in other genes: *TGFB2* (Lindsay et al., 2012), *TGFB3* (Matyas et al., 2014), Mothers Against Decapentaplegic, Drosophila, Homolog of 3 (*SMAD3*; van de Laar et al., 2011), all components of TGF-β signaling, have been found associated to other diseases (LDS1, 2 and 3, respectively) in differential diagnosis with LDS, MFS and other inherited connective tissue disorders. Patients with mutations in *TGFB2* and *TGFB3* genes display toes contractures, while patients carrying mutations in *SMAD3* present digits contractures (**Table 1**).

CONCLUSION

In conclusion, a lack of knowledge exists on the organization of EF in tendons, in particular fibrillin structure and function, and on the clinical manifestations associated to alterations of EF

in tendons, therefore this subject needs further investigation by the scientific community. Direct and indirect data suggest that tendons are affected in heritable connective tissue disorders and may play an important role in clinical features.

AUTHOR CONTRIBUTIONS

BG and GP: conception and design; writing the article; critical revision of the article; final approval of the article.

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frontiers in Aging Neuroscience

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Activation of EphA4 and EphB2 Reverse Signaling Restores the Age-Associated Reduction of Self-Renewal, Migration, and Actin Turnover in Human Tendon Stem/Progenitor Cells

*Cvetan Popov, Julia Kohler and Denitsa Docheva**

Experimental Surgery and Regenerative Medicine, Department of Surgery, Ludwig Maximilians University, Munich, Germany

Tendon tissues, due to their composition and function, are prone to suffer age-related degeneration and diseases as well as to respond poorly to current repair strategies. It has been suggested that local stem cells, named tendon stem/progenitor cells (TSPCs), play essential roles in tendon maintenance and healing. Recently, we have shown that TSPC exhibit a distinct age-related phenotype involving transcriptomal shift, poor self-renewal, and elevated senescence coupled with reduced cell migration and actin dynamics. Here, we report for the first time the significant downregulation of the ephrin receptors EphA4, EphB2 and B4 and ligands EFNB1 in aged-TSPC (A-TSPC). Rescue experiments, by delivery of target-specific clustered proteins, revealed that activation of EphA4- or EphB2-dependent reverse signaling could restore the migratory ability and normalize the actin turnover of A-TSPC. However, only EphA4-Fc stimulation improved A-TSPC cell proliferation to levels comparable to young-TSPC (Y-TSPC). Hence, our novel data suggests that decreased expression of ephrin receptors during tendon aging and degeneration limits the establishment of appropriate cell-cell interactions between TSPC and significantly diminished their proliferation, motility, and actin turnover. Taken together, we could propose that this mechanism might be contributing to the inferior and delayed tendon healing common for aged individuals.

Keywords: tendon aging and degeneration, TSPC, ephrins, cell migration, actin dynamics

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Cesare Faldini,
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Reviewed by:
Takao Sakai,
University of Liverpool, UK
Monika Lucia Bayer,
University of Copenhagen, Denmark

****Correspondence:***
Denitsa Docheva
Denitsa.Docheva@med.uni-muenchen.de

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INTRODUCTION

Tendon injuries due to excessive mechanical stress or tissue aging and/or degeneration are common and present a significant challenge for orthopedic surgery. Aged and/or degenerated tendons respond poorly to classical medicinal treatments, which often leads to rupture reoccurrence. Until now, several major factors contributing, directly, or indirectly, to tendon aging and degeneration were identified: disturbance of extracellular matrix turnover; decreasing cell numbers and metabolic activity; tenocyte dedifferentiation; and depletion or senescence of the local stem/progenitor cell pool (Jozsa and Kannus, 1997; Kaeding and Best, 2009; Zhou et al., 2010; Kohler et al., 2013).

Tendon stem/progenitor cells (TSPCs) were first reported in (Bi et al., 2007) as plastic adherent cells that possess strong clonogenic potential and express classical stem cell markers, while maintaining the expression of typical tendon-lineage genes, such as Scleraxis and tenomodulin (Bi et al., 2007; Kohler et al., 2013). Additionally, these cells are able to differentiate to three different lineages *in vitro* and more importantly, can form tendon-like tissue *in vivo* (Bi et al., 2007). In the same year, by using chimeric tendon-GFP rat models, (Kajikawa et al., 2007) proposed that tendon healing is carried out mainly by such local tendon progenitor cells, which actively migrate to the wound site and engage in cell proliferation. However, others and we have found that TSPC features alter during tendon aging and degeneration (Zhou et al., 2010; Kohler et al., 2013). Aged TSPC (A-TSPC) display a profound self-renewal deficit accompanied with premature entry into senescence and substantial changes in their transcriptome, especially in genes regulating cell adhesion, migration and cytoskeleton, but unaltered multipotential (Kohler et al., 2013). Furthermore these cells exhibit severely dysregulated cell–matrix interactions, motility and actin dynamics (Kohler et al., 2013).

In recent years, the role of ephrins and their signaling in regulating numerous cellular processes has been recognized in different cell types and tissues. Ephrins are receptor tyrosine kinases that mediate short-range cell-cell communication. For their activation, a binding between the membrane-bound ephrin receptor (Eph) and ephrin ligand (EFN) located on the surface of the neighboring cell is required. There are 9 EphA and 5 EphB receptors, and 5 EFNA and 3 EFNB ligands expressed in humans. Typically, EphA receptors bind to EFNA ligands and EphB receptors bind to EFNB ligands; however, EphA4 and EphB1 receptors can bind to both EFNA and EFNB ligands (Egea and Klein, 2007). Eph-EFN bond initiate simultaneously bidirectional signaling in the receptors (forward) and ligands (reverse) expressing cells that can activate key cellular kinases, such as focal adhesion kinase (FAK), extracellular signal-regulated kinases (ERK), Akt, c-Jun N-terminal kinases (JNKs) and p38 mitogen-activated protein kinases (p38), and thereon can influence cell self-renewal, migration, and actin turnover (Murai and Pasquale, 2003; Pasquale, 2010; Arthur et al., 2011).

Ephrin receptor-ligand interactions and their diverse roles have been the best studied in neuronal and cancerous cells (Genander and Frisen, 2010). Ephrins were linked to regeneration of the central neuronal system due their functions in neuronal connections and axon guidance (Du et al., 2007). Certain ephrin members have been associated with cancer cell migration and tumor progression (Guo et al., 2006).

Few studies have also elaborated on the role for ephrins and their signaling in musculoskeletal tissues. For example in bone, ephrins can positively influence osteoblast differentiation (Zhao et al., 2006; Xing et al., 2010), but suppress osteoclastogenesis by affecting TRAP, cathepsin K and integrin β3 signaling (Zhao et al., 2006; Cheng et al., 2012; Stiffel et al., 2014). In muscle development, ephrin interactions are required for the appropriate formation of neuromuscular junctions, nerve branching and topographic innervation within individual muscles, as well as for myoblast directed migration to the dorsal and ventral limb muscles (Swartz et al., 2001; Stark et al., 2011). Interestingly, it has been found that ephrins can also affect the functions of tissue-resident stem cells. Arthur et al. (2011) reported that activation of EFNB1 and EFNB2 reverse signaling inhibit the attachment and spreading of bone-marrow mesenchymal stem cells, while stimulation of EphB2 and EphB4 forward signaling promotes their migration. Goichberg et al. (2011) and Li and Johnson (2013) showed that EphA2/EFNA1 and EFNA5 can enhance the migration of human and bovine cardiac stem cells, correspondingly. In a follow up study, Goichberg et al. (2013) found that disturbed EphA2/EFNA1 signaling is related to age-associated senescence and reduced migration of human cardiac progenitor cells, and demonstrated that overexpression of EphA2 in these cells can rescue their senescent and migratory phenotype.

Despite of the broader knowledge in other tissues, up-to-date ephrin family expression and functions in tendon tissues and cells essentially remain unknown. Only one developmental paper, based on *in situ* hybridization analyses, has reported during embryonal chick development a strong EphA4 expression within the tendon core (D'Souza and Patel, 1999). Interestingly, our previous microarray data suggested differential expression levels of several ephrin members in A-TSPC in comparison to the control young-TSPC (Y-TSPC; Kohler et al., 2013).

Cumulatively, this strongly motivated us to examine for the first time the expression pattern of this neglected family in TSPC. Our main aims in this study were: (i) to characterize the ephrin expression profile of TSPC *in vitro*; (ii) to identify key candidates among the expressed ephrin members that are dysregulated in A-TSPC versus Y-TSPC; and (iii) to investigate if the selected ephrin members can contribute to restoring their aging phenotype.

MATERIALS AND METHODS

Cell Isolation and Culture

The isolation and complete characterization of human Y- and A-TSPC was reported in Kohler et al. (2013, under Ethical grant No. 166-08 LMU Medical Faculty). In brief, TSPC were obtained from non-ruptured Achilles tendons from four young and 12 elder human donors as tendon tissue biopsies were minced into small pieces, enzymatically digested with 0.15% collagenase II (Worthington, USA) in culture medium at 37°C overnight, filtered with sterile nylon mesh (100 μm pore size) and centrifuged at 500 *g* for 10 min. Then, cell pellets were suspended and expanded in DMEM/Ham's F-12 (1:1 mixture) supplemented with stabile glutamine, 1× MEM amino acids (all from Merck Millipore, USA), 10% FBS, and 1% L-ascorbic acid-2-phosphate (both from Sigma–Aldrich, Germany), and the TSPC were cultivated in a humidified incubator at constant 37°C and 5% CO_2. During the initial cultivation, all individual cell lines were monitored for cell yield, morphology and expansion kinetics. Since within each group, the cell lines were very comparable, equal group size of three representative donor-derived TSPC were formed in order to carry out in-depth ephrin analyses. All experiments were performed with TSPC at passages 4–6.

Activation of ephrin-dependent signaling in A-TSPC was done based on (Kaneko and Nighorn, 2003; Arthur et al., 2011) protocol with minor modifications. First, 1 μg/ml recombinant human ephrin-Fc chimera (EphA4, EphB2, EphB4, and EFNB1-Fc, all R&D systems, USA) or control-Fc proteins were clustered with polyclonal anti-human Fc antibody (molar ratio 5:1, Dianova, Germany) in complete culture media at room temperature for 30 min. Then, A-TSPC were harvested from tissue culture dishes, counted, resuspended, and incubated in ephrin-Fc or Fc-control media for 30 min in humidified incubator. Thereafter, cells were plated for various experiments and the treated A-TSPC were supplemented every second day with fresh media, containing 1 μg/ml clustered ephrin-Fc and Fc-control. In each experiment, Y-TSPC at the same passage number was used as positive control.

Quantitative PCR Analysis

RNA isolation and cDNA synthesis were performed similar to (Popov et al., 2011): RNA was extracted with RNeasy Mini Kit (Qiagen, Gemany) and 1 μg total RNA was used for cDNA synthesis with AMV First-Strand cDNA Synthesis Kit following the manufacturer's instructions (Thermo scientific, USA). Quantitative PCR analysis for the complete ephrin family expression in human Y- and A-TSPC was done with RealTime Ready Custom panel array (Roche, Germany). Each array consisted of 9 EphA (1–8 and 10), 5 EphB (1–4 and 6), 5 EFNA (1–5), and 3 EFNB (1–3) genes and three housekeeping genes GAPDH, HPRT, and SDHA. For calculation of the relative gene expression, ratio between targeted gene and GAPDH was made. Data consisted of three independent repeats with all three Y- and A-TSPC donors ($n = 9$).

Cytochemistry

Y- and A-TSPC plated and cultured on 20 μg/ml collagen 1-coated glass slides (BD Bioscience, USA) for 48 h were fixed with 4% paraformaldehyde (Merck, Germany) or 7% formalin/PHEM (6 mM PIPES, 25 mM HEPES, 10 mM EGTA, 3 mM MgCl2, pH 6.1, all Sigma–Aldrich) solutions. Then, cells were permeabilized with 0.1% Triton X100 (Sigma–Aldrich) and blocked with 3% BSA (Millipore, USA). Primary antibodies against EphA4 (Abnova, Germany), EphB2 (Biomol, Germany), EphB4 (Thermo scientific), EFNB1 and EFNB2 (both Sigma–Aldrich) were applied overnight at 4°C. Next, secondary Alexa Flour 488-conjugated antibodies and DAPI were used (all Life technologies). For negative control, cell-seeded slides were incubated only with the secondary antibody and DAPI. For F-actin staining, formalin/PHEM fixed cells were incubated with phalloidin-AF546 (Life technologies) for 40 min at room temperature. Photomicrographs were taken with Axiocam MRm camera on AxiovertS100 microscope (Carl Zeiss, Germany). Staining experiments were repeated two independent times for all three Y- and A-TSPC donors ($n = 6$).

Western Blot Analysis

Total protein from Y- and A-TSPC (clustered EphA4 and EphB2, and Fc-control) was isolated with RIPA-buffer (0.1% SDS, 1% Na-DOC, 1% Triton X-100, 50mM Tris-HCl pH8.2, 150mM NaCl, 10mM EDTA, 20mM NaF, 1mM Na_3VO_4) supplemented with complete protease inhibitors (Roche). Proteins (20 μg) were separated on SDS gels, transferred onto PVDF membrane and blocked with 5% skim milk (Merck) for 1 h at room temperature. Primary antibodies against human EphA4 (Abnova), EphB2 (Biomol), EphB4 (Thermo scientific), EFNB1 and EFNB2 (both Sigma–Aldrich); phospho-FAK (Thermo scientific), total FAK, total and phospho-ERK1/2; total and phospho-Akt; total and phospho-p38; total and phospho-Jnk (all Cell Signaling, USA), and GAPDH (Merck) were applied overnight at 4°C. Then, membranes were incubated with corresponding secondary HRP-conjugated antibodies (Cell Signaling) for 1 h at room temperature and consequently with ECL solution (GE Healthcare, USA). Photomicrographs were taken on ImageQuant LAS 4000 mini (GE Healthcare) as band intensities were quantified with ImageProPlus4 software program (Media Cybernetics, USA). Western blot experiments were preformed two independent times with all three Y- and A-TSPC donors ($n = 6$).

Self-Renewal Analysis

Tendon stem/progenitor cell self-renewal was assessed with WST-1 proliferation and colony forming unit (CFU) assays as described in (Kohler et al., 2013). For cell proliferation, 3×10^3 cells/ cm^2 of Y- and A-TSPC (EphA4-Fc, EphB2-Fc, and Fc-control) were plated in complete culture medium containing 0.2% FBS, with or without clustered EphA4 and EphB2 protein for 7 days. Thereafter, WST-1 reagent (Roche) was applied for 4 h and optical density (OD) was measured at 420 and 620 nm using a Microtiter Reader (Thermo Scientific). Cell proliferation was calculated in percentage to Y-TSPC. WST-1 assay was reproduced three independent times with all Y- and A-TSPC donors, as each experiment was done in triplicates ($n = 9$). For the CFU assay, 20 cells/ cm^2 of Y-, and A-TSPC (EphA4-Fc, EphB2-Fc, and Fc-control) were plated in 10 cm culture dishes for 12 days in complete media supplemented with or without EphA4 and EphB2 clustered protein. Then, cell colonies were visualized with 0.5% crystal violet/methanol staining and counted. CFU efficiency was estimated as percentage of counted colonies to the number of plated cells. CFU experiment was performed three independent times of all Y- and A-TSPC donors in duplicates ($n = 9$).

Migration Analysis

Migration analysis was performed similarly to (Kohler et al., 2013) using Axiocam ICc3 camera mounted on Axiovert S100 inverted microscope (Carl Zeiss) equipped with biochamber (PeCon, Germany) providing stable culture conditions. For random migration, 1.5×10^3 cells/ cm^2 of Y- and A-TSPC (EphA4-Fc, EphB2-Fc, and Fc-control) were seeded in 6-well plates and incubated for 2 h prior imaging. Time-lapse was performed with four frames per h for 2 days. The image data was extracted with AxioVisionLE software (Carl Zeiss) and individual cell tracks were analyzed with ImageJ V1.48 software. Random migration was expressed by calculation of

the forward migration index [FMI; the ratio of the vector length to the migratory starting point based on (Sip et al., 2014)]. Results of random TSPC migration consist of three independent repeats with all Y- and A-TSPC donors ($n = 9$; total of 150 tracks per donor type). For the scratch assay, 1×10^4 cells/ cm^2 of Y- and A-TSPC (EphA4-Fc, EphB2-Fc, and Fc-control) were plated in 6-well dishes and let to form confluent monolayers for 2 days. Prior imaging, the layers were scratched multiple times. Time-lapse was performed with four frames per h for 3 days. The initial scratch length and time needed for gap bridging were measured and used for calculation of cell velocity. The scratch assay was reproduced three independent times for each donor group as each experiment was done in triplicates ($n = 9$, 36 scratches per donor type).

Quantification of Cell Area and Actin Dynamics

Quantification of Y- and A-TSPC (EphA4-Fc, EphB2-Fc, and Fc-control) cell area was obtained from F-actin images by measuring 50 cells from each donor with the polygonal tool of the ImageProPlus4 software (Media Cybernetics). Actin dynamics of Y- and A-TSPC (EphA4-Fc, EphB2-Fc, and Fc-control) was analyzed by latrunculin A experiment, carried out as described in (Kohler et al., 2013). Latrunculin A inhibits actin polymerization by sequestering monomeric G-actin and thereby disrupts the turnover of actin filaments. In brief, the four different TSPC groups, Y- and A-TSPC with EphA4-Fc, EphB2-Fc, and Fc-control, (5.5×10^3 cells/cm^2) were grown in 96 well plates and pre-cultured for 48 h. Then, cells were treated with 0.4 μM latrunculin A (Sigma–Aldrich), fixed at different time intervals (0, 2, 5, 8, 10, 20, 30, and 60 min), stained with 0.6 μM phalloidin-546 (Thermo Scientific) and fluorescence signals were recorded at 573 nm using a SAFIRE2 microplate reader (Tecan, Germany). In each group, F-actin content at time point 0 was set to 100%. Latrunculin A analyses were reproduced three independent times for all Y- and A-TSPC donors in triplicates ($n = 9$).

Statistical Analysis

In the study 3 Y-TSPC and 3 A-TSPC representative lines were used in all experiments. The quantitative data was generated out of all three different donors per group and furthermore each donor was used in 3 ($n = 9$) or 2 ($n = 6$) independent experiments. Statistical evaluation was performed using the GraphPrism software (GraphPad, La Jolla, CA, USA). Final graphs and bar charts show mean values ± SD of two or three independent experiments for the donor group. Within each of the independent experimental repeats, the individual donors were represented in triplicates, however, the replicates were calculated as mean value for the donor. Unpaired *t*-test was used for two group analysis and Tukey's one-way ANOVA was applied for multi group statistical testing. A p-value < 0.05 was considered statistically significant ($^*p < 0.05$; $^{**}p < 0.01$, $^{***}p < 0.001$).

RESULTS

Expression Levels of Several Ephrin Members were Significantly Downregulated in A-TSPC

We first compared the whole ephrin family expression profile in Y- and A-TSPC by using quantitative PCR (**Figure 1A**). Our results demonstrated that in comparison to Y-TSPC, A-TSPC expressed significantly lower levels of EphA4 (3.5 folds), EphB2 (7 folds), and EphB4 (7 folds) receptors, and EFNB1 (1.3 folds) ligand, but increased levels of EFNB2 (2.3 folds). These expression changes were further verified on protein level by immunocytochemical staining (**Figure 1B**) and western blotting (**Figure 1C**), which confirmed the downregulation of EphA4, EphB2, EphB4, and EFNB1, and upregulation of EFNB2 expression in A-TSPC. Taken together, our novel data evidently showed that during tendon aging and degeneration, EphA4, EphB2, EphB4, and EFNB1 mRNA and protein expression levels are significantly downregulated in TSPC.

Analysis of Activation of Key Cellular Kinases in A-TSPC Selects EphA4 and EphB2 as Main Candidates of Interest

In order to investigate the importance of EphA4, EphB2, EphB4, and EFNB1 downregulation for TSPC aging and degeneration, we next designed rescue experiments based using established protocols (refer to Materials and Methods). We first stimulated externally A-TSPC with EphA4, EphB2, EphB4, and EFNB1 clustered proteins and then analyzed the activation of their downstream signaling in the cells (**Figure 2A**). As mentioned in the introduction, the main downstream effectors of ephrin-mediated signaling are the cellular kinases FAK, ERK, Akt, JNK, and p38 (**Figure 2B** and **Supplementary Figure S1**). Direct comparison in-between basal kinase activities demonstrated that ERK and Akt phosphorylation was significantly elevated in A-TSPC. EphA4-Fc stimulation of A-TSPC led to increased FAK and JNK activity and more importantly, to reduced ERK phosphorylation to levels comparable to Y-TSPC. Stimulation with EphB2-Fc resulted in significantly increased phosphorylation levels of JNK and p38 kinases in A-TSPC. These results suggests that EphA4 and EphB2 signaling overlaps mainly in activation of JNK kinase; however, these both ephrins demonstrated clear difference in ERK, FAK, and p38 activation. In comparison to EphA4 and EphB2, activation with EphB4-Fc and EFNB1-Fc did not show robust effect on A-TSPC kinase activity. Therefore, in our next experiments, we continued only with EphA4-Fc and EphB2-Fc, and investigated their effect on A-TSPC self-renewal, migration and actin dynamics (refer to **Figure 2C**).

EphA4-Fc Signaling, but not EphB2-Fc, Positively Affects A-TSPC Self-Renewal

We investigated the effect of EphA4-Fc and EphB2-Fc on TSPC self-renewability by performing short-term cell proliferation (WST-1; **Figure 3A**) and colony-forming units (CFU) assays

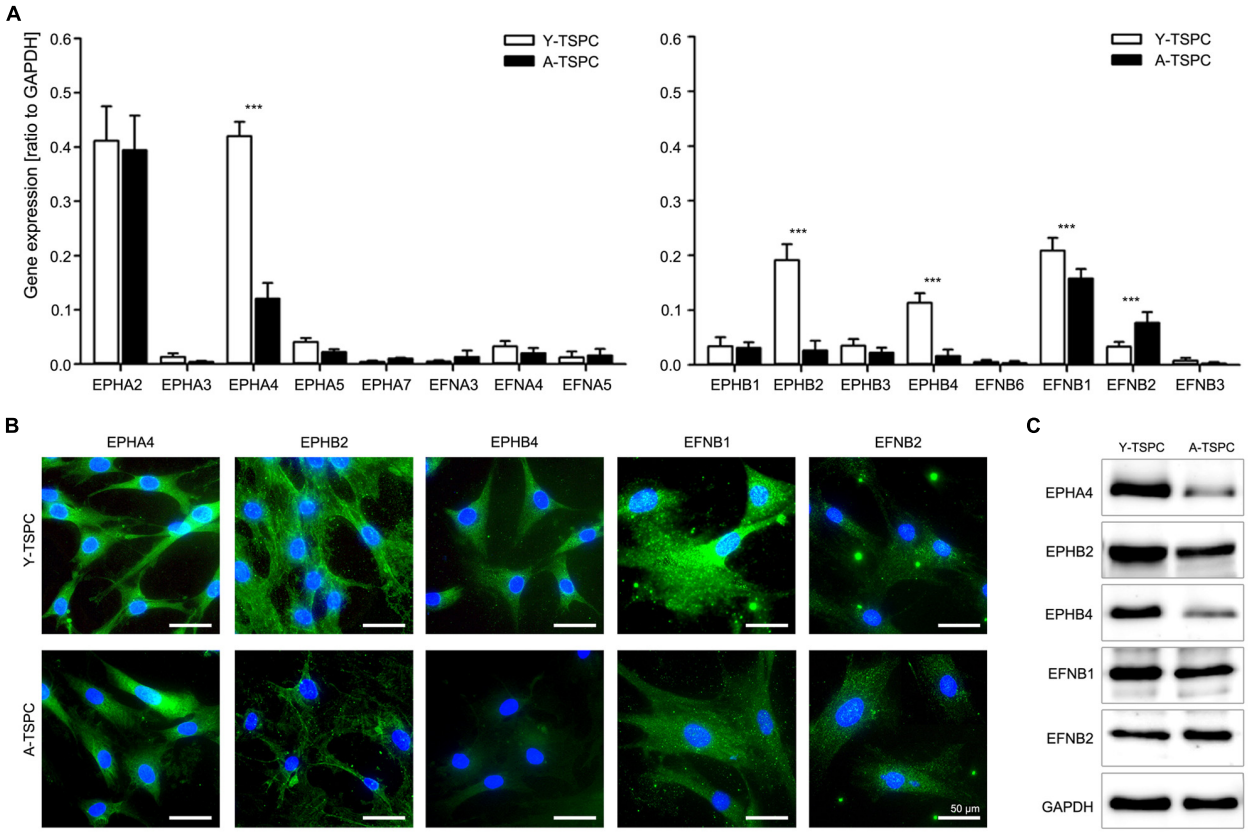

FIGURE 1 | Ephrin expression in tendon stem/progenitor cells (TSPCs). (A) Comparison between Y- and A-TSPC basal ephrin family expression by quantitative PCR array. Data consists of three independent repeats with all three Y-and A-TSPC donors ($n = 9$, $^{***}p \leq 0.001$). **(B)** Cytochemical and **(C)** western blot protein analysis for EphA4, EphB2, EphB4, EFNB1, and EFNB2 in Y- and A-TSPC ($n = 6$; full blot shown in **Supplementary Figure S1**). Representative images; bars = 50 µm.

(**Figure 3B**). A-TSPC exhibited slower proliferation rate and clonogenic ability. Interestingly, when treated with clustered EphA4-Fc A-TSPC proliferation significantly increased to levels comparable to Y-TSPC, while supplementation with EphB2-Fc had no effect. The addition of EphA4-Fc to A-TSPC had tendency to elevate their CFU numbers; however, it did not lead to significant change ($p = 0.06$). In comparison to EphA4-Fc, the second candidate EphB2-Fc showed a significant difference as treatment with this ephrin inhibited the A-TSPC clonogenic potential with approximately 12-folds, thus suggesting that EphA4- and EphB2-mediated reverse signaling cascades diverge in their cellular functions.

EphA4-Fc and EphB2-Fc Restore the Migration Deficit of A-TSPC

In order to determine whether EphA4-Fc and EphB4-Fc have positive influence, we next performed random migration and scratch assays (**Figure 4**). The random migration, represented by FMI confirmed that A-TSCP are indeed less migratory than Y-TSPC and showed that the addition of both EphA4-Fc and EphB2-Fc enhances their cell motility (**Figure 4A**). Moreover, quantification of accumulated length and velocity clearly indicated that the observed effect is significant (**Figure 4B**). However, in-between both types of receptors, EphA4 was more potent inducer of TSPC migration. This finding was further validated by scratch assay (**Figures 4C,D**) revealing that EphA4-Fc A-TSPC closed first the distance between the two fronts (13.2 ± 0.9 h), followed by Y-TSPC (15.4 ± 1.3 h), EphB2-Fc A-TSPC (17.4 ± 0.5 h), and last A-TSPC (20.9 ± 0.6 h) (**Figure 4C**). In summary, we can report for the first time that activation of EphA4 or EphB2-dependent reverse signaling can rescue the migration deficit of A-TSPC, furthermore, EphA4-Fc stimulated A-TSPC performed even better than Y-TSPC.

EphA4-Fc and EphB2-Fc Improve A-TSPC Actin Dynamics

Finally, we performed phalloidin staining for F-actin and compared the actin filament dynamics in the four TSPC groups by treating them with latrunculin A in a time-dependent manner. First, we investigated changes in cell area and we found neither EphA4-Fc nor EphB2-Fc affected the cell area habituated by A-TSPC (**Figure 5A**). In contrast, our latrunculin a analyses, performed 48 h after A-TSPC treatment with the corresponding ephrins, indicated reduced F-actin content

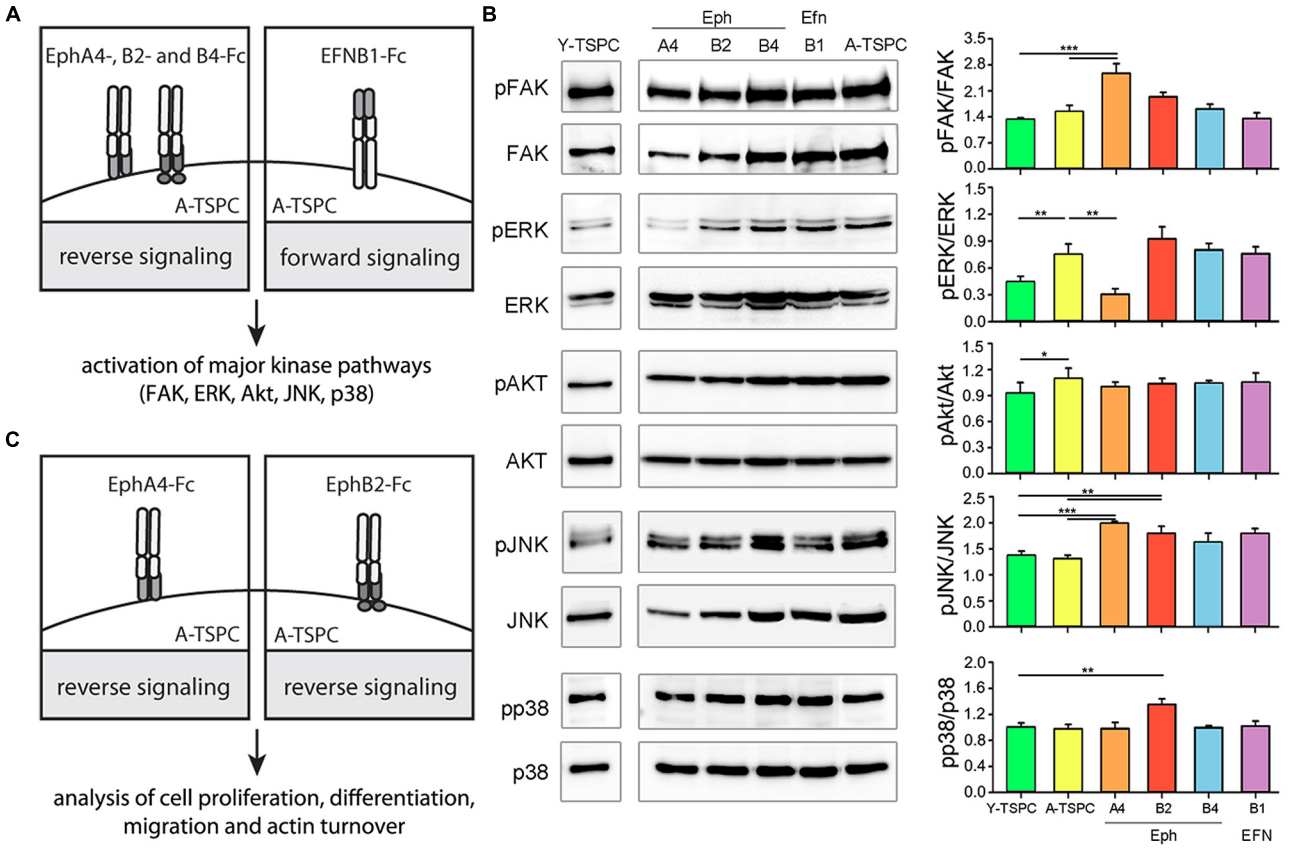

FIGURE 2 | Experimental design and western blot analysis of key cellular kinases in TSPC. (A) Schematic presentation of A-TSPC stimulation with clustered ephrins. **(B)** Western blot analysis of phosphorylated and total FAK, ERK, Akt, JNK, and p38 kinases in Y-TSPC and EphA4-Fc, EphB2-Fc, EphB4-Fc, EFNB1-Fc, and control-Fc A-TSPC. Data consists of two independent repeats with all three Y-and A-TSPC donors ($n = 6$, $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$). **(C)** Experimental study design.

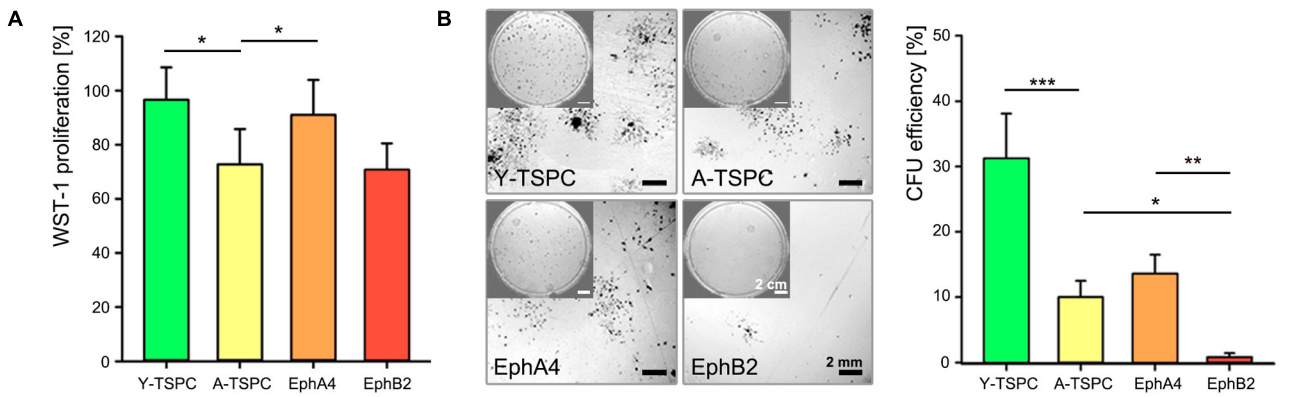

FIGURE 3 | Self-renewal potential of EphA4-Fc and EphB2-Fc A-TSPC. (A) WST-1 proliferation and **(B)** CFU analysis of Y-TSCP, EphA4-Fc, EphB2-Fc, and control-Fc A-TSPC. Data consists of three independent repeats with all three Y-and A-TSPC donors ($n = 9$, $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$).

visualized by phalloidin staining at 60 min (**Figure 5B**). Quantification of time-dependent F-actin decrease and curve declination angle (**Figure 5C**) showed that both EphA4-Fc and EphB2-Fc significantly improved the actin turnover of A-TSPC to levels comparable to Y-TSPC. **Figure 5D** represents the basal levels of F-actin in the four different groups prior the latrunculin A treatment (upper graph, time point 0 min). However, the EphA4-Fc- or EphB2-Fc-treated A-TSPC, when challenged with

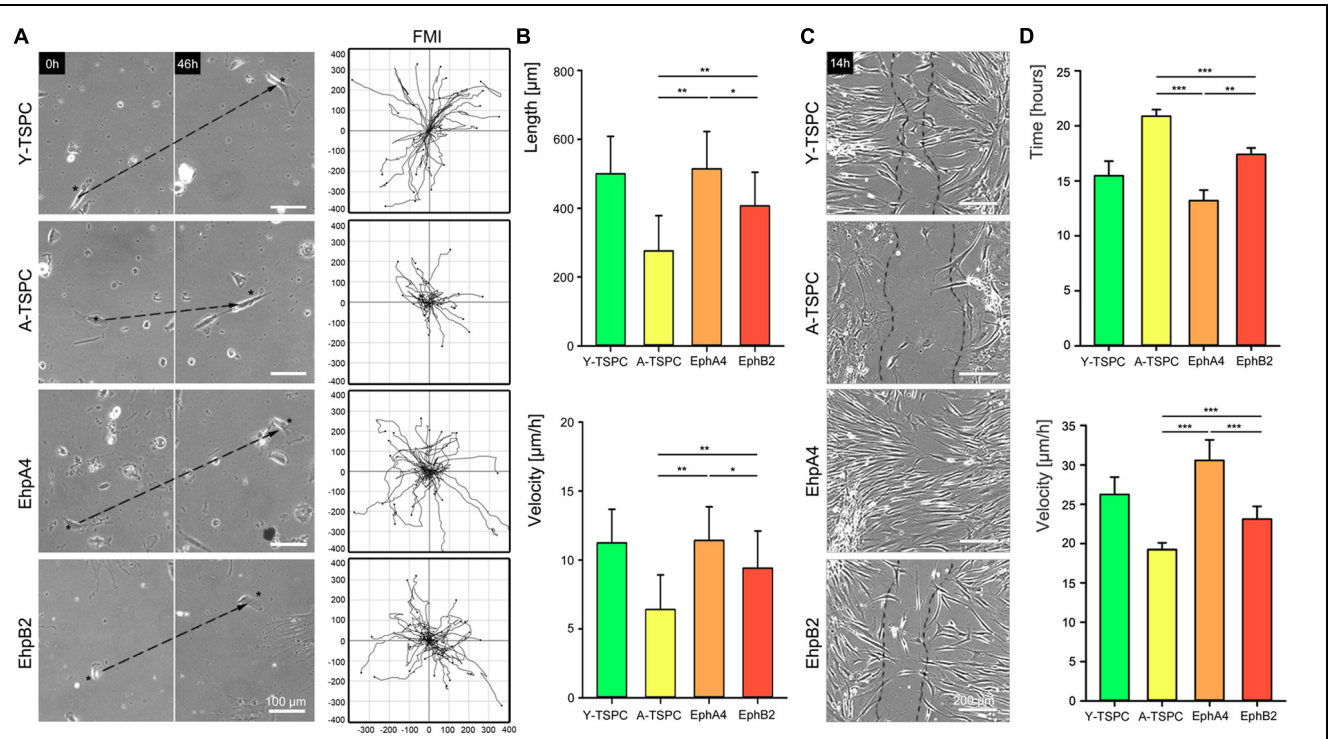

FIGURE 4 | Migration of EphA4-Fc and EphB2-Fc A-TSPC. (A) Representative images of random migration (dash lines show ecludian distance) and calculation of forward migration index (FMI). Bars = 100 µm. **(B)** Quantification of random migration (distance and velocity) ($n = 9$, total of 150 tracks per donor type). **(C)** Representative scratch assay of Y-TSCP, EphA4-Fc, EphB2-Fc, and control-Fc A-TSPC. Bars = 200 µm. **(D)** Estimation of scratch bridging time and cell velocity ($n = 9$, total of 36 scratches per donor type, $^*p < 0.05$; $^{**}p < 0.01$, $^{***}p < 0.001$).

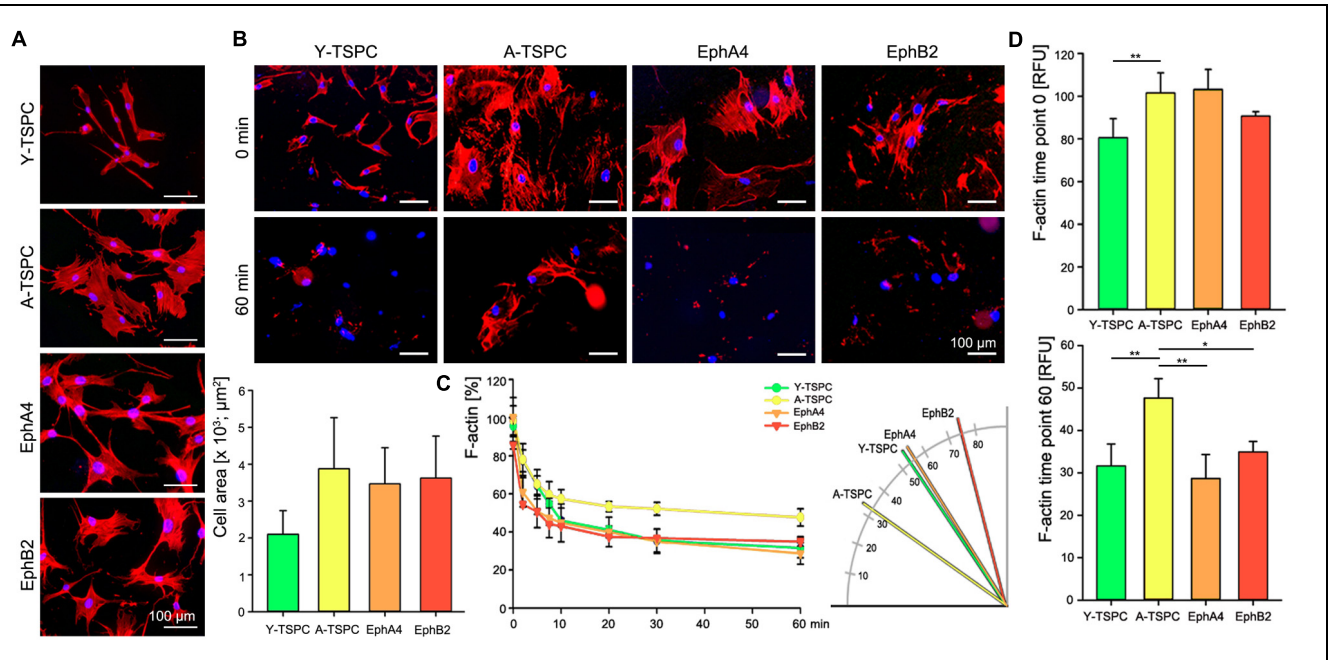

FIGURE 5 | Actin dynamics of EphA4-Fc and EphB2-Fc A-TSPC. (A) Representative images of F-actin staining and quantification of the cell area of Y-TSPC, EphA4-Fc, EphB2-Fc, and control-Fc A-TSPC ($n = 9$, total of 50 cells per donor). **(B)** Representative images of Y-TSPC, EphA4-Fc, EphB2-Fc, and control-Fc A-TSPC at seven time points during latrunculin A treatment. **(C)** Quantification of actin filament dynamics in a time-dependent manner and curve declination angle. **(D)** Total actin content at the beginning (0 min) and at the end (60 min) of the latrunculin A experiment ($n = 9$, $^*p < 0.05$; $^{**}p < 0.01$).

LatA, were able to normalize their F-actin content to levels similar to Y-TSPC (lower graph, time point 60 min).

In summary, our novel findings demonstrate that activation of EphA4- and EphB2-dependent reverse signaling augments the motility and actin turnover of A-TSPC, but only EphA4 rescues their cell proliferation deficit.

DISCUSSION

In the current study, we report for the first time that A-TSCP have dysregulated cell–cell interactions mediated by the ephrin family. By comparing Y- to A-TPSC we found that the expression of several ephrin members is significantly changed. Next, by carefully examining the role of two main candidates, namely the receptors EphA4 and EphB2, we could demonstrate that by activating their reverse signaling, via external delivery of the clustered receptors, we can normalize several of the A-TSCP deficits.

The importance of ephrin receptor-ligand interactions has increasingly been recognized not only in the neuronal system (Du et al., 2007) and cancer (Guo et al., 2006), but also in musculoskeletal tissues such as bone (Zhao et al., 2006; Cheng et al., 2012; Stiffel et al., 2014) and muscle (Swartz et al., 2001; Stark et al., 2011). As mentioned in the introduction, only one paper so far reported on the expression of EphA4 during tendon morphogenesis in chick limbs (D'Souza and Patel, 1999). Recent study focusing on periodontal ligament fibroblasts and osteoblasts showed important strain-dependent involvement of EphB4/EFNB2 in modulating osteogenesis during tooth movement (Diercke et al., 2011). Hence, to our knowledge we are the first to report on the ephrin expression profile in TSPC. Among the 15 Eph receptors and the nine EFN ligands found in human, TSPC expressed predominantly four receptors and two ligands. Furthermore, upon tendon aging and degeneration, EphA4, EphB2, and EphB4 and EFNB1 ligand were significantly downregulated, while EFNB2 was upregulated, both on mRNA and protein levels. After initial screening of the downregulated ephrin candidates, we decided to focus in the main part of our study on rescue experiments with EphA4-Fc and EphB2-Fc proteins and to investigate their effect on the self-renewal, migration and actin dynamics of A-TSPC.

We started with self-renewal analyses. Previous literature on different ephrin members has suggested strong cell-specific effects; for example, EphA7/EFNA2 negatively regulates the cell proliferation of adult neural progenitor in the olfactory bulb (Holmberg et al., 2005), whereas EphB2-forward signaling stimulates intestine progenitor self-renewal (Genander et al., 2009). When we examined TSPC self-renewal by performing short-term cell proliferation and CFU assays, we found that EphA4-Fc, but not EphB2, can rescue the age-associated drop of proliferation as well as demonstrated a tendency to elevate A-TSPC clonogenicity, a finding which can be further explored and optimized in follow up studies. Our investigation of the downstream effectors revealed that both clustered proteins trigger activation of JNK kinase, however, EphA4-Fc also elevates the FAK phosphorylation, while EphB2 p38 phosphorylation. Hence, we speculate that this divergence in their signaling cascades might be contributing for the different effect on A-TSPC self-renewal; however, follow up studies will be necessary to investigate in great details the exact molecular mechanisms.

Next, we focused on investigating the effect of EphA4-Fc and EphB2-Fc on A-TSPC migration and actin turnover. We have described the age-related marked reduction in cell motility and actin dynamics of TSPC in (Kohler et al., 2013); here, we report for the first time that both EphA4-Fc or EphB2-Fc can significantly increase A-TSPC random migration and wound healing to rates similar to, and in the case of EphA4-Fc even better than, that of Y-TSPC. This novel data is in line with several studies claiming a positive effect of different ephrin family members on stem or progenitor cell migration (Arthur et al., 2011; Goichberg et al., 2011; Li and Johnson, 2013). Actin cytoskeletal dynamics are pivotal for cell migration (Rottner and Stradal, 2011), therefore by implementing latrunculin A experiments and F-actin staining we compared the cell shapes, F-actin content and the kinetic, and speed of actin filament turnover in Y-TSPC versus A-TSPC with or without EphA4-Fc and EphB2-Fc. Our results clearly demonstrated that activation of EphA4 or EphB2 reverse signaling in A-TSPC significantly improves their actin turnover to a mode similar to Y-TSPC. However, the A-TPSC shape and F-actin amount at the start point of the experiments were not affected by the addition of EphA4-Fc and EphB2-Fc, a finding that is different to other reports showing a regulatory function of certain ephrins on cell shape and spreading (Stokowski et al., 2007; Yamazaki et al., 2009; Arthur et al., 2011). It has been proposed that ephrins can affect cell migration by interfering with actin cytoskeleton dynamics via modulated GTPase activity (Marston et al., 2003; Buricchi et al., 2007; Takeuchi et al., 2015). A current limitation of our study is the lack of specification of EphA4 and EphB2 reverse partners in TSPC. Based on the expression analyses, we can speculate that the major ligands are EFNB1 or EFNB2, however, the identification of the exact EphA4 or EphB2-dependent reverse signaling pathway, and the possible involvement of GTPases, remain as challenges to be addressed in future research.

Taken together, our novel data suggests that decreased expression of the ephrin receptors EphA4 and EphB2 during tendon aging and degeneration limits the establishment of appropriate cell–cell interactions between TSPC and significantly diminished their proliferation, motility and actin turnover. We found that delivery of EphA4-Fc or EphB2-Fc to A-TSPC can successfully normalize several of the key aspects of their age-related phenotype and that in-between the two the EphA4-Fc is the more dominant. We could propose that dysregulation in EphA4-trigered bi-directional signaling may contribute to the inferior and delayed tendon healing common for aged individuals, which will be the focus for upcoming investigation. Hence, we believe that our pioneering study provides important and novel insight into the complex nature of tendon aging and degeneration.

AUTHOR CONTRIBUTIONS

CP and DD contributed to analysis and interpretation of data and design of the work, CP and JK contributed to acquisition of data; CP and DD were involved in drafting of the work and in the work revision; all authors were involved in the final approval of the manuscript and are agreeing to be accountable for all aspects of the work and ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fnagi.2015.00246

FIGURE S1 | Complete western blot image. Aliquots of equal amounts of protein from three Y-TSPC and A-TSPC treated with EphA4-, EphB2-, EphB4-, and EFNB1-Fc proteins were loaded onto the same western blot membranes, which were first probed for the expression levels of phospho-FAK, ERK, AKT, Jnk, and p38. Next, the same membranes were stripped and probed for the corresponding total-FAK, ERK, AKT, Jnk, and p38. Comparable protein loading was validated with anti-GAPDH antibody. Complete western blot image corresponding to the phosphor-lanes in the inset B of **Figure 2**. The same procedure was performed for another two A-TSPC donors. Abbreviations: MW – positions of the molecular weight protein standard; 1–3, different Y-TSPC donors; A4, EphA4-Fc-; B2, EphB2-Fc-; B4, EphB4-Fc; B1, EFNB1-Fc-treated, and "–", non-treated A-TSPC.

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ORIGINAL RESEARCH
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Patterns of Age-Associated Degeneration Differ in Shoulder Muscles

Yotam Raz[1,2], Jan F. Henseler[1], Arjen Kolk[1], Muhammad Riaz[3], Peer van der Zwaal[4], Jochem Nagels[1], Rob G. H. H. Nelissen[1] and Vered Raz[3]*

[1] Department of Orthopaedics, Leiden University Medical Center, Leiden, Netherlands, [2] Department of Molecular Epidemiology, Leiden University Medical Center, Leiden, Netherlands, [3] Department of Human Genetics, Leiden University Medical Center, Leiden, Netherlands, [4] Department of Orthopaedic Surgery, Medical Center Haaglanden, Hague, Netherlands

Shoulder complaints are common in the elderly and hamper daily functioning. These complaints are often caused by tears in the muscle-tendon units of the rotator cuff (RC). The four RC muscles stabilize the shoulder joint. While some RC muscles are frequently torn in shoulder complaints others remain intact. The pathological changes in RC muscles are poorly understood. We investigated changes in RC muscle pathology combining radiological and histological procedures. We measured cross sectional area (CSA) and fatty infiltration from Magnetic Resonance Imaging with Arthrography (MRA) in subjects without ($N = 294$) and with ($N = 109$) RC-tears. Normalized muscle CSA of the four RC muscles and the deltoid shoulder muscle were compared and age-associated patterns of muscle atrophy and fatty infiltration were constructed. We identified two distinct age-associated patterns: in the supraspinatus and subscapularis RC muscles CSAs continuously declined throughout adulthood, whereas in the infraspinatus and deltoid reduced CSA was prominent from midlife onwards. In the teres minor, CSA was unchanged with age. Most importantly, age-associated patterns were highly similar between subjects without RC tear and those with RC-tears. This suggests that extensive RC muscle atrophy during aging could contribute to RC pathology. We compared muscle pathology between torn infraspinatus and non-torn teres minor and the deltoid in two patients with a massive RC-tear. In the torn infraspinatus we found pronounced fatty droplets, an increase in extracellular collagen-1, a loss of myosin heavy chain-1 expression in myofibers and an increase in Pax7-positive cells. However, the adjacent intact teres minor and deltoid exhibited healthy muscle features. This suggests that satellite cells and the extracellular matrix may contribute to extensive muscle fibrosis in torn RC. We suggest that torn RC muscles display hallmarks of muscle aging whereas the teres minor could represent an aging-resilient muscle.

Keywords: muscle atrophy, aging, shoulder, rotator cuff tear, fatty infiltration, MRA, fibrosis, satellite cells

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Luciano Merlini,
Istituto Ortopedico Rizzoli, Italy

Reviewed by:
Guo-Yuan Yang,
Shanghai Jiao Tong University, China
Ibrahim Mahjneh,
University of Oulu, Finland

****Correspondence:***
Yotam Raz
y.raz@lumc.nl

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INTRODUCTION

Musculoskeletal disorders are highly prevalent in the elderly, leading to substantial hindering of functional mobility and daily functioning. Over half of the individuals above the age of 70 develop chronic shoulder diseases (Picavet and Schouten, 2003). Despite the high impact of RC pathology on daily functioning in the elderly, the effect of aging on the shoulder muscles is poorly understood (Hermans et al., 2013). In previous studies a strong correlation was found between the presence of a RC-tear and age, suggesting that RC muscles are under continuous age-associated stress (Feng et al., 2003; Fehringer et al., 2008; Yamamoto et al., 2010). However, how muscle degeneration in the shoulder changes during aging in the intact RC, as well as in RC-tears remains unclear. Ninety percent of these shoulder complaints are either diagnosed as subacromial pain syndrome (SAPS) or as tears of the stabilizing rotator cuff (RC; Steinfeld et al., 1999; Koester et al., 2005). Although past research on RC-tear mainly focused on the tendons, recently muscle degeneration was also considered to play a causative role (Laron et al., 2012). However, pathophysiology of the RC muscles in tear conditions are poorly understood.

Muscle atrophy, defined by the loss of muscle mass, is associated with loss of muscle strength and increase in fatty infiltration and inflammation (Evans, 2010). Muscle atrophy is highly prominent in the elderly and can distinguish between healthy and frail individuals (Taekema et al., 2012). Muscle atrophy in RC-tears is considered as a clinical determinant for surgical success in RC-tear repair (Tashjian et al., 2010; Mall et al., 2014) and long-term functionality after surgery (Shen et al., 2008). Recently, atrophy of the supraspinatus (SSp) RC muscle has been suggested to have a prominent role in RC-tearing (Barry et al., 2013). The SSp is the foremost affected RC muscle, and so far is the major focus of most studies (Nakagaki et al., 1996; Ashry et al., 2007; Barry et al., 2013). Atrophy of the infraspinatus (ISp) RC muscle was also suggested to contribute in RC diseases (Henseler et al., 2015). As the interplay between all four RC muscles coordinates shoulder movements and stability, it is crucial to consider all four RC muscles to understand the pathogenesis of RC-tears. A description of muscle atrophy and fatty infiltration in the RC could be constructed from non-invasive radiological imaging (Shen et al., 2008; Mall et al., 2014). How muscle pathology changes with age in all RC muscles was not reported. In aging muscles, changes in muscle mass and muscle strength are accompanied by histological changes. Histological changes in torn muscles, albeit frequent in the aging population, are not well-studied. Histological features of aging muscles include extra-cellular matrix (ECM) thickening and fatty infiltration (Brack et al., 2007; Zoico et al., 2010). Whether those pathological marks are also exhibited in torn RC muscles is not fully understood.

The objective of this study was to assess muscle degeneration in both patients with intact and torn RC muscles. Muscle atrophy and fatty infiltration, obtained from Magnetic Resonance imaging with Arthrography (MRA) were used as measures for muscle pathology. We compared patterns of muscle atrophy between subjects without a RC-tear and subjects with a RC-tear for all four RC muscles: SSp, subscapularis (SSc), ISp, and teres minor (Tmi); and the adjacent deltoid (Del). Furthermore, marks of muscle aging were investigated in histological staining comparing torn ISp to non-torn Tmi and Del. Our study suggests that aging-associated histopathological changes differ in skeletal muscles and suggests the Tmi as an aging-resilient muscle.

MATERIALS AND METHODS

Study Design and Participants

A retrospective cross-sectional study was performed on a consecutive series of shoulder MRAs at the orthopedics outpatient clinics in the Medical Center Haaglanden hospitals in the Netherlands between January 1, 2012 and February 13, 2013 ($N = 442$). All patients with atraumatic and chronic shoulder complaints or shoulder instability are routinely evaluated with MRA. Ethical approval was obtained from the Medical Ethics Committee of the Landsteiner Institute, Medical Center Haaglanden for the radiologic evaluations. Since the radiologic evaluations pertain to a retrospective study, the Medical Ethics Committee waived the need for informed consent from the participants included in this study. Four hundred and forty-two shoulder MRAs were identified. Exclusion was based on poor image quality ($N = 21$), presence of a tumor ($N = 5$), isolated biceps tears ($N = 4$), subscapularis tears ($N = 3$), and fractures ($N = 6$). Subjects were grouped according to the absence ($N = 294$) or presence of a RC-tear ($N = 109$) on shoulder MRA. In total, 403 MRAs are included in this study. The RC-tear group included 40 partial SSp tears (53.5 $\pm$ 9.5 years old), 57 full thickness SSp tears (54.7 $\pm$ 11.7 years old), five full-thickness SSp tears with partial detachment of the ISp tears (63.2 $\pm$ 9.6 years old) and seven full-thickness SSp and ISp tears (61.0 $\pm$ 9.1 years old). Excluded from the analyses were: 12 images with motion artifacts of the SSc and 29 images with an incomplete field of view of the Del muscle.

Muscle biopsies were collected from two patients with a massive RC-tear of the SSp and the ISp. During tendon transfer surgeries (Henseler et al., 2013, 2014) muscle biopsies of the ISp, Tmi, and Del were obtained. Radiological characteristics of these two patients are detailed in **Table 4**. Medical Ethical approval was obtained from the Medical Ethical Committee of the Leiden University Medical Center for the collection and analyses of the biopsies and informed consent was obtained from the patients involved.

MRA Imaging Procedure

Fifteen minutes before MRA, contrast fluid was injected under fluoroscopic guidance into the glenohumeral joint from posterior. All MRAs were performed on Avanto or Symphony MRI units (Siemens AG, Erlangen, Germany) using a dedicated shoulder coil and turbo spin-echo sequences.

Analyses of the images were performed on a PACS Workstation with Sectra IDS5 (Sectra Medical Systems AB,

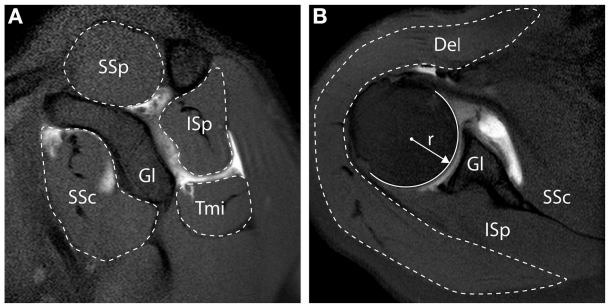

FIGURE 1 | Measurements of muscle cross-sectional surface area from MR Arthography. (A) Sagittal view. Cross-sectional surface areas (CSA) of the supraspinatus (SSp), infraspinatus (ISp), teres minor (Tmi), and subscapularis (SSc), relative to the glenoid (Gl) are depicted. **(B)** Transversal view. CSA of the deltoid (Del) is depicted. All CSAs were normalized to the surface of the humeral head, calculated based on the radius of the humeral head (*r*).

Linköping, Sweden) as monitor readings. As multiple planes and sequences were obtained following the institutional standard shoulder MRA protocol, the T1-weighted transversal and sagittal plane (TR/TE 500-600/11-15, matrix 256; slice thickness 4 mm, inter-slice gap 1 mm, field of view of 15 cm) were systematically evaluated.

Muscle cross sectional area (CSA) quantification, was described previously (Henseler et al., 2015), and examples are shown in **Figure 1**. In brief, the radius (*r*) of the humeral head at the widest point was measured from its widest point using a circle fit in the transversal plane, and is reported in millimeters (mm). The RC muscles (i.e., SSp, SSc, ISp, Tmi) CSA were measured from the sagittal slice with the anatomical glenoid neck and base of the coracoid present, as illustrated in **Figure 1A**, and reported in mm^2. The Del was measured from the transversal slice with the humeral head at its widest point, as illustrated in **Figure 1B**, and reported in mm^2. Muscle CSA was normalized to the humeral head surface (in mm^2), in order to correct for inter-individual anthropometric differences.

The presence of fatty infiltration of the RC was evaluated by examining the presence of intramuscular fatty infiltration in the SSp, SSc, ISp, and Tmi muscles on the sagittal T1-weighted images, and was scored according to the Goutallier score (1, no fatty infiltration; 2, <50% fatty infiltration; 3, about 50% fatty infiltration; 4, more than 50% fatty infiltration; Goutallier et al., 1994).

Muscle Biopsies

During operation biopsies were collected from the Del, ISp, and Tmi muscles and were immediately frozen in liquid nitrogen and stored at −80°C. Cryosections (16 μm thick) were made with the CM3050-S cryostat (Leica, Solms, Germany) on dry ice and pasted on Superfrost plus glass slides (Menzel-Gläser, Braunschweig, Germany). Sections were stored at −20°C prior to staining. Histological procedures included: (1) Gomori-Trichrome staining (Gomori, 1950). (2) Immunostaining for collagen with goat-collagen-type I (1:200, SouthernBiotech, Birmingham, Alabama, USA), and for satellite cells with mouse-Pax7 antibodies (1:75, Developmental Studies Hybridoma Bank (DSHB), Iowa City, Iowa, USA). Primary antibodies were detected with secondary anti-goat-Alexa-594 or anti-mouse-Alexa-488 (1:1000, Molecular Probes, Invitrogen, Waltham, Massachusetts, USA), respectively. (3) Nile red (1 μM, Sigma-Aldrich, Saint Louis, Missouri, USA) detecting fatty droplets. (4) Immunostaining for myosin heavy chain (MyHC) isotypes was carried out in two sequential steps: first tissues were incubated with monoclonal antibodies detecting MyHC-2x (1:5, hybridoma 6H1, DSHB) and laminin (1:1000, Abcam, Cambridge, UK), followed by secondary anti-mouse-Alexa-546 or anti-rabbit-Alexa-647 (1:1000, Molecular Probes), respectively. Subsequently tissues were incubated with a mixture of monoclonal antibodies to MyHC-1 and MyHC-2a [hybridoma BA-D5 and SC-71, respectively (DSHB)], which are conjugated to Alexa-350 and Alexa-594, respectively, washed and mounted. Conjugation was carried out as previously described (Gregorevic et al., 2008). All sections were treated on the same day with the same antibody mix. Slides were mounted with Aqua Polymount (Polyscience, Niles, Illinois, USA). Nuclei were counterstained with DAPI. Images were generated with either the DM5500 (fluorescent) or DMLB (light) microscopes (Leica, Wetzlar, Germany) using LAS AF software V2.3.6, and for the MyHC immunostaining with the Array scan VTI HCA (Thermofisher, Waltham, Massachusetts, USA). For quantification of Pax7 positive cells, imaging was carried out with a 40X objective.

Statistical Analyses

Differences in characteristics between subjects without or with RC-tears were evaluated with independent *t*-tests and χ^2-tests. Age-association of CSA was carried out on standardized scores of the normalized CSA. Standardization was performed on the group without and with RC-tears separately. Correlations between standardized scores of the normalized CSA and fatty infiltration (Goutallier score) were evaluated with Pearson correlation tests. Correlations were performed within and in between muscles. Age distribution of subjects with and without RC-tear and in RC-tear shows both a normal distribution and therefore a simple linear regression model corrected for gender was applied to assess age-associated changes. The beta (β) and Pearson correlation coefficient (*R*) were calculated. Visualization of age-related trends in standardized CSA and fatty infiltration is provided in four age groups for subjects without tears, and similar age groups in RC-tear. Statistical significance was considered with a $p < 0.05$ (two-sided). Statistical analyses were performed with SPSS Statistics (IBM Inc., Armonk, New York, USA).

RESULTS

Subject Characteristics

Muscle CSA and fatty infiltration were measured in five shoulder muscles from 403 individuals. Subject characteristics were stratified for diagnosis (without or with RC-tear), as the mean age in RC-tear was significantly higher compared to those without

TABLE 1 | Characteristics of subjects.

	Without RC-tear RC-tear (*N* = 294)	RC-tear (*N* = 109)	*p*-values
DEMOGRAPHIC DATA			
N	294	109	
Age (years)	42.1 (14.3)	55.1 (10.8)	<0.001
Female, *N* (%)	115 (39.1)	40 (36.7)	0.73
RADIOGRAPHIC DATA			
Surface head of humerus (mm^2)	1954 (383)	1940 (366)	0.74
SSp MUSCLE			
Normalized CSA	0.81 (0.21)	0.60 (0.26)	<0.001
Without fatty infiltration, *N* (%)	232 (78.9)	46 (42.2)	*<0.001
With fatty infiltration, *N* (%)	62 (21.1)	63 (57.8)	
SSc MUSCLE			
Normalized CSA	1.62 (0.76)	1.43 (0.64)	0.01
Without fatty infiltration, *N* (%)	208 (73.2)	41 (37.6)	*<0.001
With fatty infiltration, *N* (%)	76 (26.8)	68 (62.4)	
ISp MUSCLE			
Normalized CSA	1.17 (0.32)	0.99 (0.35)	<0.001
Without fatty infiltration, *N* (%)	249 (84.6)	61 (56.0)	*<0.001
With fatty infiltration, *N* (%)	45 (15.2)	48 (44.0)	
Tmi MUSCLE			
Normalized CSA	0.71 (0.21)	0.71 (0.25)	0.97
Without fatty infiltration, *N* (%)	279 (94.9)	91 (83.5)	*0.001
With fatty infiltration, *N* (%)	15 (5.1)	18 (16.5)	
Del MUSCLE			
Normalized CSA	6.17 (1.68)	6.24 (1.61)	0.70

Means (SD) are provided unless otherwise stated. Muscle cross-sectional area (CSA) is normalized to the humeral head surface. Fatty infiltration was assessed according to the Goutallier classification score, shown are the number and (%) of patients without (Goutallier 1) or with fatty infiltration (Goutallier 2,3,4). Supraspinatus (SSp), Subscapularis (SSc), Infraspinatus (ISp), Teres minor (Tmi), Deltoid (Del).

Nominal variables between subjects without RC-tear and RC-tear patients were compared with t-tests.

$^*\chi^2$-tests were used to compare fatty infiltration across the Goutallier scores between subjects without RC-tear and RC-tear patients.

RC-tear (**Table 1**). CSAs of the SSp, ISp, and SSc muscles were significantly lower, and in all five muscles fatty infiltration was significantly higher in the RC-tear group compared with the group without RC-tear.

Correlation Between Muscle Atrophy and Fatty Infiltration

We assessed the correlation between a decrease in muscle CSA and an increase in fatty infiltration as a robust measure for muscle degeneration. Within subjects without RC-tear a significant correlation was found only for the SSp and in the SSc muscles (**Table 2A**). However, in the RC-tear group, significant correlations were found for all four RC muscles (**Table 2B**). A decrease in CSA of the SSp correlated with increase in fatty infiltration in the other three RC muscles both in the group without RC-tear and in RC-tears (**Tables 2A,B**). Additionally in the RC-tear group, a decrease in the CSA of the ISp correlated with an increase in fatty infiltration in the other three RC muscles (**Table 2B**).

Age-Association of Muscle Atrophy and Fatty Infiltration

Age-associated trends of muscle atrophy and fatty infiltration were assessed using a linear regression model, adjusted for gender. In subjects without RC-tears, age-associated decline of SSp and SSc CSA was significant (**Table 3**). In the SSp and SSc muscles the CSA decreased constantly between 14 and 85 years (**Figure 2Ai**). The CSA decline in the SSp was 2.6-fold higher compared to that in the SSc. In contrast, in the ISp, Tmi, and Del a decline in muscle CSA was only found from midlife onwards (**Figure 2Ai**). Fatty infiltration showed an age-associated increase in all five muscles (**Table 3**), however it was most prominent in the older age group (61–85 years; **Figure 2Aii**).

In the RC-tear group, an age-associated decline in muscle CSA was found in the SSp, SSc, ISp, and Del muscles, whereas the Tmi CSA was unaffected (**Table 3**). In this RC-tear group the SSp muscle was consistently torn in all individuals. However, the decline in the muscle CSA was comparable between the SSp, SSc, and ISp (**Table 3**). Same as in the non-tear group, also in the RC-tear group CSA declined continuously throughout adulthood, whereas in the ISp and Del it started after midlife (**Figure 2i**). Moreover, in the RC-tear group an age-associated increase in fatty was found in all five muscles (**Table 3**, **Figure 2ii**). Overall, a decline in muscle CSA and an increase in fatty infiltration were both more pronounced in the RC-tear group as compared with the group without RC tear. However, the age-associated patterns were similar between the two groups: a continuous decline in muscle CSA was found for SSp and SSc, but in the ISp and Del the decline started only from midlife onwards (**Table 3**, **Figure 2**).

Histological Analyses for Muscle Degeneration

To explore whether the radiological features in RC-tear conditions are accompanied by aging-associated tissue degeneration, we performed histological analyses of muscle biopsies with known aging histopathological marks. Muscle biopsies were obtained from two subjects at comparable age. Both patients had a massive RC tear involving the SSp and ISp. Their radiological characteristics were more severe than the average of the entire RC tear group (**Table 4**). CSA of SSp and ISp from Patient A were more than 1.5 standard deviations (SD) smaller than the mean of the RC-tear group of the radiological study (**Table 4**). However, CSA of these muscles from Patient B were within 1 *SD* of the mean of the RC-tear group (**Table 4**). Overall patient A had more severe muscle atrophy in all five muscles compared to patient B. Histological staining of the torn ISp from both patients showed severe disruption of myofiber orientation, accompanied with fibrosis and fat cells (**Figure 3A**). In the non-torn Tmi from both patients, fibrosis and fat cells were less prominent compared with the ISp (**Figure 3A**). In the Del muscle histology was not pathological (**Figure 3A**). We confirmed extensive fibrosis and thickening of the ECM in the ISp using collagen-1 immunostaining in both patients (**Figure 3B**). However, ECM thickening was limited in the Tmi and Del from both patients (**Figure 3B**). This suggests ECM thickening is among the pathological hallmarks of torn RC muscles.

TABLE 2 | Correlations of muscle cross sectional area and fatty infiltration in the four RC muscles.

Muscle CSA \ Fatty infiltration	*SSp*		*SSc*		*ISp*		*Tmi*	
	Pearson correlation	*p*-values	Pearson correlation	*p*-values	Pearson correlation	*p*-values	Pearson correlation	*p*-values
(A) WITHOUT RC-TEAR (*N* = 294)								
SSp	**−0.33**	**<0.001**	**−0.27**	**<0.001**	**−0.22**	**<0.001**	**−0.20**	**0.001**
SSc	−0.08	0.17	**−0.27**	**<0.001**	−0.11	0.07	−0.01	0.92
ISp	−0.06	0.34	−0.08	0.17	−0.09	0.12	−0.04	0.53
Tmi	−0.05	0.38	−0.02	0.72	−0.06	0.32	−0.11	0.06
(B) RC-TEAR (*N* = 109)								
SSp	**−0.66**	**<0.001**	**−0.42**	**<0.001**	**−0.50**	**<0.001**	**−0.23**	**0.02**
SSc	−0.18	0.06	**−0.42**	**<0.001**	**−0.23**	**0.02**	−0.18	0.07
ISp	**−0.62**	**<0.001**	**−0.34**	**<0.001**	**−0.67**	**<0.001**	**−0.35**	**<0.001**
Tmi	0.01	0.95	−0.13	0.19	0.04	0.65	**−0.29**	**0.003**

Muscle degeneration is assessed by the Pearson correlation between standardized-scores of normalized muscle cross sectional area (CSA) and Goutallier scores of fatty infiltration for each muscle. Panel A shows analyses in subjects without RC-tear; Panel B shows analyses in RC-tear. SSp, Supraspinatus; SSc, Subscapularis; ISp, Infraspinatus, Tmi, Teres minor. Pearson correlations and p-values are provided. Statistically significant correlations are depicted in bold. Correlations between muscle CSA and fatty infiltration within the same muscle are depicted in red.

TABLE 3 | Age-associated analyses of muscle cross sectional area and fatty infiltration in five muscles of subjects without RC-tear and in RC-tear.

Muscle	Radiological parameter	Without RC-tear (*N* = 294)			RC-tear (*N* = 109)		
		Beta for age	*R*	*p*-values	Beta for age	*R*	*p*-values
SSp	Muscle CSA	**−0.021 (0.004)**	**0.38**	**<0.001**	**−0.030 (0.008)**	**0.34**	**0.001**
	Fatty infiltration	**0.018 (0.002)**	**0.51**	**<0.001**	**0.034 (0.009)**	**0.35**	**<0.001**
SSc	Muscle CSA	**−0.008 (0.004)**	**0.17**	**0.04**	**−0.026 (0.009)**	**0.38**	**0.003**
	Fatty infiltration	**0.024 (0.003)**	**0.48**	**<0.001**	**0.037 (0.007)**	**0.45**	**<0.001**
ISp	Muscle CSA	−0.003 (0.004)	0.25	0.5	**−0.031 (0.008)**	**0.4**	**<0.001**
	Fatty infiltration	**0.014 (0.002)**	**0.46**	**<0.001**	**0.031 (0.008)**	**0.34**	**<0.001**
Tmi	Muscle CSA	−0.003 (0.004)	0.18	0.39	−0.015 (0.009)	0.19	0.09
	Fatty infiltration	**0.006 (0.001)**	**0.34**	**<0.001**	**0.018 (0.006)**	**0.32**	**0.001**
Del	Muscle CSA	−0.001 (0.122)	0.27	0.81	**−0.023 (0.008)**	**0.43**	**0.007**

Linear regression analysis was performed to identify age-dependent changes in standardized-scores of normalized muscle cross sectional area (CSA) or in fatty infiltration. The models are adjusted for gender. SSp, Supraspinatus; SSc, Subscapularis; ISp, Infraspinatus; Tmi, Teres minor, Del, Deltoid. Beta (± standard errors) and Pearson correlation coefficient (R) are provided. Statistically significant models are depicted in bold.

We validated fatty infiltration using Nile-red staining. This confirmed fatty infiltration in the ISp from both patients, but only limited fatty droplet staining was found in Tmi or Del muscles (**Figure 3C**).

To determine contractile features of those muscles we employed immunostaining for three MyHC isotypes. Laminin staining was used to identify myofiber contour. Laminin staining revealed disruption in myofiber orientation in the torn ISp (**Figure 3D**). Additionally, we found that while both MyHC-1 and -2a, were expressed in Del muscles, in the Tmi and the torn ISp the expression of MyHC-1 was dramatically reduced. Furthermore, MyHC-2x was co-expressed with MyHC-2a in the torn ISp of patient B (**Figure 3D**).

We also explored the regenerative capacity of torn RC muscles. Muscle sections were immunostained with an anti-Pax7 antibody, marking satellite cells. We found a two- to three-fold increase in the fraction of Pax7-positive nuclei in torn ISp compared to Del or Tmi (**Figures 4A,B**). The fraction of Pax7-positive nuclei between Del and Tmi was similar (**Figure 4B**). Furthermore, in the Del and Tmi all Pax7 staining overlaid within myonuclei, whereas in the torn ISp Pax7 staining was also found outside myonuclei (**Figure 4A**).

Overall, histological analyses confirmed that in both patients the torn ISp is severely degenerated compared to the non-torn Tmi and Del muscles. Some histological differences were found between the two patients, especially with MyHC isotypes expression. Overall in both patients histological features of the Tmi RC muscle were comparable to Del muscles rather than to ISp RC-muscle.

DISCUSSION

Aging-associated changes in skeletal muscles are prominent in part because it is the most abundant in the human body. There

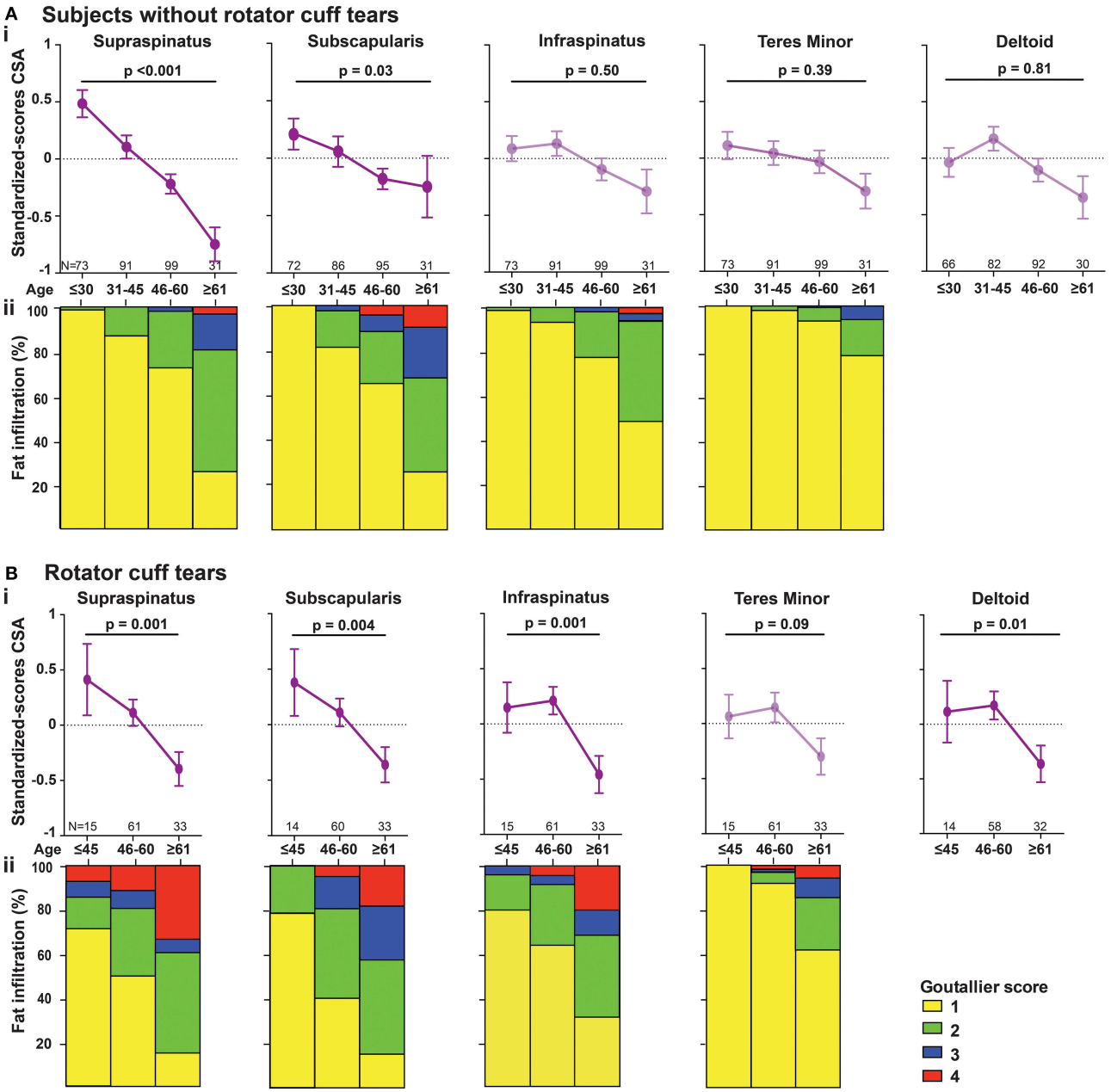

FIGURE 2 | Age-associated changes in muscle cross sectional area and fatty infiltration in shoulder muscles. Age-associated analyses were performed in five shoulder muscles (supraspinatus, subscapularis, infraspinatus, teres minor, and deltoid) in subjects without RC-tear **(A)** and in RC-tear **(B)**. (**i**) Age-associated trends of standardized muscle cross-sectional surface areas. *p*-values for age-association were calculated using linear regression and are adjusted for gender. Significant trends are depicted in dark purple, non-significant trends are depicted in light purple. (**ii**) Age-associated increase in fatty infiltration. Fatty infiltration was evaluated according to the Goutallier score.

are over 400 skeletal muscles in the human body and how their pathology changes during aging is largely unknown. So far most studies are carried out on vastus lateralis using cross-sectional studies. In vastus lateralis an aging-associated decline in muscle strength and muscle mass starts at the sixth decade (Williams et al., 2002; Faulkner et al., 2007). Here we found that muscle atrophy in ISp and Del muscles starts only after the age of 45, while a continuous decline throughout adulthood was found in the SSp and SSc. This indicates that mechanisms leading to muscle atrophy could be similar between vastus lateralis, ISp and Del muscles, but are likely to differ from those regulating muscle atrophy in SSp and SSc. In contrast, in the Tmi, muscle atrophy did not change significantly with age, suggesting this muscle is less susceptible for age-associated changes. Since the Tmi is

TABLE 4 | Radiological characteristics of subjects for histology.

	Patient A (64 years)	Patient B (62 years)
SSp MUSCLE		
Normalized CSA (St)	0.21 (–1.52)	0.36 (–0.97)
Goutallier score	2	2
SSc MUSCLE		
Normalized CSA (St)	0.83 (–0.92)	1.01 (–0.66)
Goutallier score	1	1
ISp MUSCLE		
Normalized CSA (St)	0.22 (–2.15)	0.74 (–0.71)
Goutallier score	3	1
Tmi MUSCLE		
Normalized CSA (St)	0.58 (–0.52)	0.88 (+0.67)
Goutallier score	1	1
Del MUSCLE		
Normalized CSA (St)	5.29 (–0.60)	9.03 (+1.70)

Muscle cross-sectional area (CSA) is normalized to the humeral head surface. St, corresponding standardized score in the entire RC-tear group. Fatty infiltration was assessed according to the Goutallier classification score.

unaffected in RC-tears (Melis et al., 2011), it is poorly studied. We suggest that the Tmi could represent an aging-resilient muscle. The histology of Tmi muscle from two patients with a massive RC-tear was also showed a healthy histology, similar to that of Del muscles within the same patient. In contrast, the torn ISp exhibited aging and degenerated muscle pathology. The Tmi muscle could be used to identify potential molecular regulators that protect skeletal muscles from damage during aging.

Torn muscles are often characterized by atrophy and fatty infiltration (Goutallier et al., 1994; Barry et al., 2013). We confirmed this increase in fatty infiltration in torn muscles using fatty droplets staining. Additionally, we found ECM thickening in torn muscles, indicating fibrosis. These features are also common in aging muscles (Brack et al., 2007; Zoico et al., 2010), suggesting that torn RC muscles and aging muscles share pathological mechanisms. However, this should be confirmed by additional studies. Changes in the contractile function is marked by the expression of MyHC isotypes, which can be changed in aging and in disease (Ciciliot et al., 2013). Fiber type transitions can vary between skeletal muscles, presumably due to different functions (Ciciliot et al., 2013). A transition from fast (type 1) to slow (type 2) myofibers is often found in muscular dystrophies and in metabolic disorders (Ciciliot et al., 2013). In the lower limb, type-2 myofibers decrease in aging vastus lateralis muscles (Verdijk et al., 2007). However, a transition from slow (type-1) to fast (type-2) myofibers is found in muscle disuse conditions, including denervation and loss of tensile strength (Ciciliot et al., 2013). The myofiber transition in torn RC is not well-characterized. In the intact RC MyHC type-1, -2a, and -2x are expressed in all four RC muscles (Lovering and Russ, 2008). Reduced MyHC-1 has been reported in cases with a severely torn SSp (Lundgreen et al., 2013). In agreement with that study, we also found a prominent loss of type-1 MyHC in torn ISp. Moreover, in severe denervation conditions (e.g., spinal cord injury) a myofiber switch from slow to fast fiber type was found (Verdijk et al., 2012) and is also consistent with our findings in torn ISp. Interestingly, the axillary nerve innervates the Tmi, but the SSp and ISp are innervated by the suprascapular nerve. Recent hypotheses suggest a role for denervation in torn RC muscles (Gigliotti et al., 2015). This calls for additional studies on the role of denervation in RC-tear and in muscles preserved from tearing.

In addition, we found that severely degenerated ISp muscles have an increased number of Pax7-positive cells. However, this increase may not represent an increase of satellite cells as some of the Pax7-staining was not within myonuclei. An increase in Pax7-positive cells was also reported in affected muscles from oculopharyngeal muscular dystrophy (OPMD; Gidaro et al., 2013). OPMD is a slow progressive myopathy, which could represent accelerated muscle aging (Raz and Raz, 2014). Satellite cells in chronic and slow progressive conditions were suggested to suppress muscle regeneration, possibly by remaining dormant and by differentiating into fibrogenic cells (Brack et al., 2007; Sciorati et al., 2015). Moreover, an adverse local environment could contribute to decreased regeneration by satellite cells (Meng et al., 2015). Although we analyzed biopsies from only two patients, as comparisons were performed within the same patient our findings likely represent degenerative changes between torn and non-torn RC muscles. Future studies with a larger sample size should investigate degenerative changes in torn RC muscles.

We also found an age association of fatty infiltration in subjects without an RC-tear however this was mostly contributed by the elderly group. Consistent with previous studies (Goutallier et al., 1995; Gerber et al., 2007; Gladstone et al., 2007), our results also show that fatty infiltration in RC-tear is highly prominent. In this cross-sectional study muscle atrophy appears at earlier age compared with fatty infiltration. This suggests that muscle atrophy in the RC develops earlier than fatty infiltration, while fatty infiltration in unaffected muscles is possibly systemic. Although our evaluation of fatty infiltration from MRA is qualitative, it is in agreement with a quantitative radiological study, where similar age-association of fatty infiltration was found in torn SSp (Nozaki et al., 2015).

Although muscle atrophy and fatty infiltration are both increased in aging how they are interrelated is not fully understood. In subjects without RC-tear we found that muscle atrophy correlated with fatty infiltration only in the SSp and SSc muscles. However, in RC-tear due to higher atrophy and fatty infiltration, correlations were found within all four RC muscles. In subjects without RC-tear we found that the SSp atrophy correlated with fatty infiltration in all other three RC muscles. Those correlations between muscle atrophy and fatty infiltration with the other three muscles were expanded to the ISp in RC-tear. This suggests that muscle atrophy in the SSp may affect muscle degeneration in the adjacent RC muscles.

Comparable trends of muscle atrophy were found in subjects without as well as with a RC-tear. Although the trends in muscle atrophy with age are similar between subjects without and with a RC-tear, the atrophy of the RC muscles overall was larger in the RC-tear group. In agreement, age-associated muscle atrophy in the SSp in both groups was reported, but was more pronounced in RC-tears (Barry et al., 2013). This suggests accelerated muscle

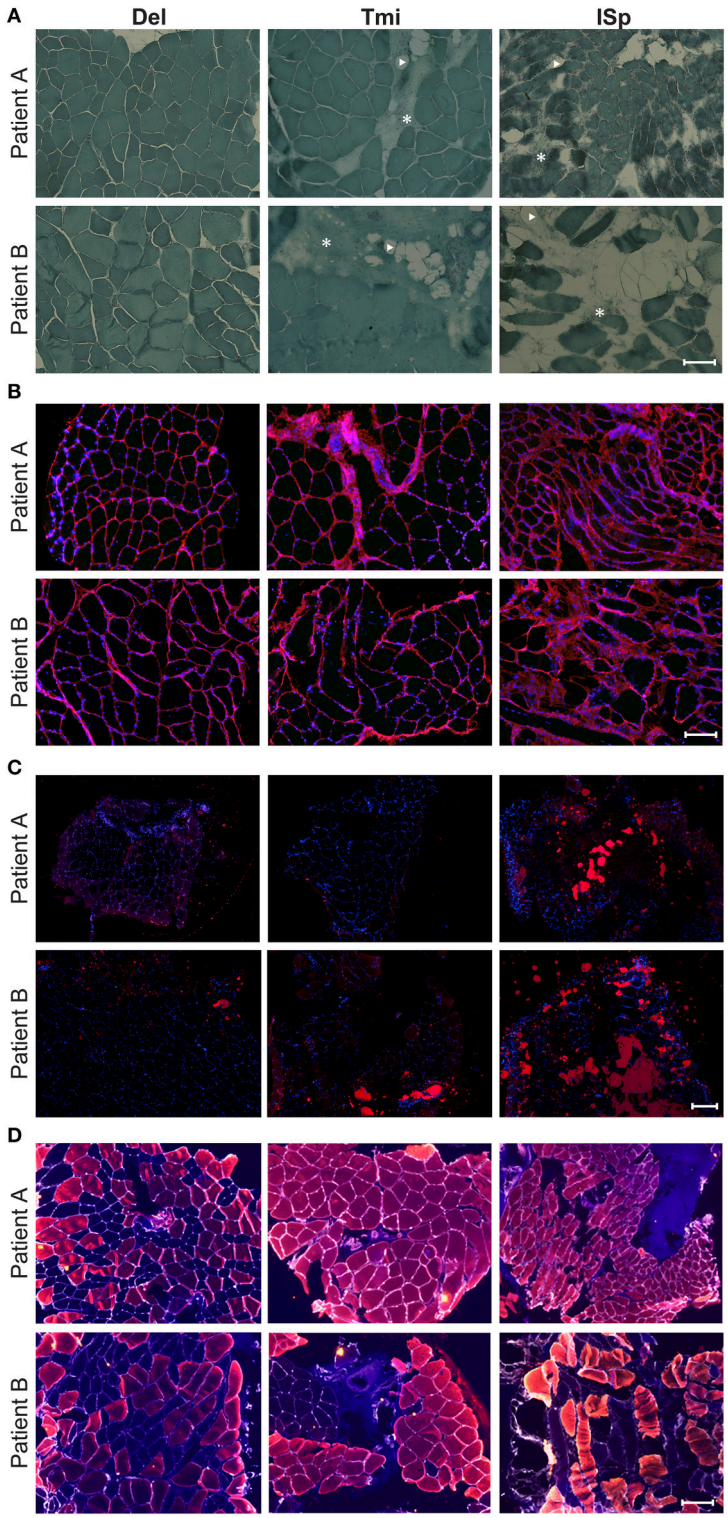

FIGURE 3 | Histological markers of muscle degeneration in torn and non-torn shoulder muscles. Representative images of histological analyses of biopsies of torn infraspinatus (ISp), non-torn RC muscle teres minor (Tmi), and deltoid (Del) in two patients. **(A)** Gomori-Trichrome staining shows myofibers in blue-green, nuclei in purple. Fat cells are negatively stained (arrow heads) and fibrotic areas are stained light blue-green (asterisks). Scale bar represents 100 μm. **(B)** Collagen immunostaining (red), nuclei are counterstained with DAPI (blue). Scale bar represents 100 μm. **(C)** Fatty droplet (red) staining, nuclei are counterstained with DAPI (blue). Scale bar represents 200 μm. **(D)** Immunostaining for myosin heavy chain (MyHC) isotypes: MyHC-1 (blue), MyHC-2a (red), MyHC-2x (yellow), and the basal lamina (gray). Scale bar represents 100 μm.

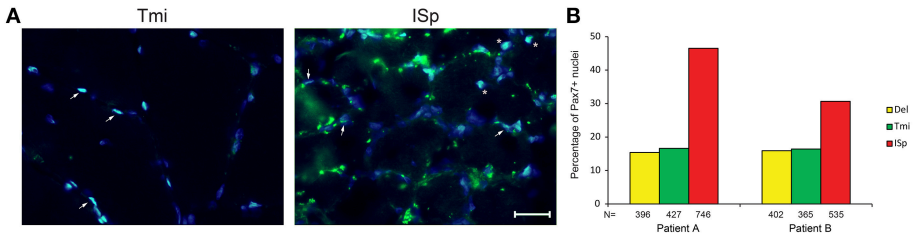

FIGURE 4 | Satellite cells in torn and non-torn shoulder muscles. (A) Representative images of Pax7 immunostaining (green) in non-torn RC muscle teres minor (Tmi) and torn infraspinatus (ISp). Nuclei were counter-stained with DAPI (blue). Pax7-positive nuclei have an overlay of blue and green. Examples of Pax7-positive myonuclei are marked with arrowheads. Examples of Pax7-positive nuclei not in myonuclei are marked with asterisks. Scale bar represents 25 μm. **(B)** Bar charts for the quantification of Pax7-positive nuclei in deltoid, teres minor and infraspinatus sections of two patients. *N* represents the number of total nuclei analyzed for each muscle in 11 fields.

atrophy in RC-tears. It is unclear whether muscle wasting in the RC is a consequence or cause of RC-tear. Longitudinal studies could further reveal the causality of muscle atrophy in RC-tear.

We conclude that patterns of age-associated degeneration differ in skeletal muscles of the RC. While some RC muscles show continuous changes throughout adulthood, in others changes start only from midlife onwards. Whereas, the majority of RC muscles show age-associated changes, the teres minor did not show significant age-associated changes. In torn RC muscles satellite cells and the ECM are increased compared to the intact teres minor. We propose that torn RC muscles display hallmarks of muscle aging whereas the teres minor could represent an aging-resilient muscle, suggesting a role of muscle pathology in RC tear pathogenesis.

AUTHOR CONTRIBUTIONS

YR and JH measured and analyzed MRA images and wrote the MS. MRA images were provided by PvdZ. Biopsies were collected by JFH, AK, and JN. Sectioning of biopsies, histological staining and imaging were performed by YR, MR, and VR. RN and VR supervised the project. All authors contributed to the writing and discussions of the results. All authors read and approved the final manuscript.

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Platelet Rich Plasma Therapy in Non-insertional Achilles Tendinopathy: The Efficacy is Reduced in 60-years Old People Compared to Young and Middle-Age Individuals

*Vincenzo Salini, Daniele Vanni, Andrea Pantalone and Michele Abate**

Orthopaedic and Traumatalogical Clinic, Department of Medicine and Science of Aging, Università degli Studi "G. d'Annunzio" Chieti-Pescara, Chieti, Italy

Background: Platelet Rich Plasma (PRP) has shown positive and long-lasting effects in patients with tendinopathies. However, information about age-related differences in the clinical outcome is limited. Aim of this retrospective study was to compare the efficacy of PRP therapy in young and elderly subjects suffering for Achilles tendinopathy.

Materials and method*:* Patients with recalcitrant non-insertional Achilles tendinopathy were enrolled. Clinical (VISA-A) and instrumental (ultrasonography) data were collected at baseline and after 1, 3, 6, and 12 months. PRP injections (once a week for 3 weeks) were performed in sterile conditions and under ultrasound (US) control.

Results: Forty-four subjects (29 young: mean age 39.5 ± 6.9; 15 elderly: mean age 61.5 ± 5.3) were retrospectively evaluated. At baseline, no significant differences were observed in the clinical and US parameters. Throughout the whole length of the study, a significant increase of VISA-A score was seen in both groups (from 50.3 ± 8.8 to 76.1 ± 6.6 in the young group, and from 48.7 ± 7.6 to 61.1 ± 9.4 in the elderly group); however, the infra-groups comparison showed better results in young patients, compared to the aged counterpart.

Conclusion: Our results show that PRP is less effective in aged people. This finding can be ascribed to several biochemical and biomechanical differences documented in tendons of young and elderly subjects (reduced number and functionality of tenocytes and tenoblasts), which becomes more evident in the long-term tissue healing. However, prospective trials, using different PRP preparations and enrolling a larger number of subjects, are needed to draw more sound and definitive conclusions.

Keywords: Achilles tendinopathy, aging, platelet rich plasma, young, ultrasonography

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Edited by:
Cesare Faldini,
Istituto Ortopedico Rizzoli, Italy

Reviewed by:
Alberto Di Martino,
University Campus Bio-Medico of Roma, Italy
Nicolás Fiz,
Arthroscopic Surgery Unit, Spain

****Correspondence:***
Michele Abate
m.abate@unich.it

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INTRODUCTION

In the last decade Platelet Rich Plasma (PRP) has been extensively used in the treatment of tendinopathies. Many trials have been performed on different tendons, and several PRP preparations and treatment schedules have been proposed. These studies, broadly speaking, have shown positive and long-lasting effects on symptoms and function in a large percentage of cases (Andia and Abate, 2012; Andia and Maffulli, 2013; Andia et al., 2014). A superiority of PRP in comparison to placebo or other usual treatments (e.g., eccentric training, physical therapies, steroid injections) has been observed by some authors, but not by others (Andia and Abate, 2012; Andia and Maffulli, 2013; Kearney et al., 2013; Andia et al., 2014).

Despite the large amount of data gathered in these experiments, information about possible difference in efficacy age-related is limited. In particular, at our knowledge, only in few papers this topic has been addressed with inconclusive results, evaluating different factors (age, sex, BMI, duration of symptoms, severity of degeneration, and others), which can theoretically influence the clinical outcomes (Ferrero et al., 2012; Boesen et al., 2014; Filardo et al., 2014).

This is an important point, because the prevalence of tendinopathies nowadays is increasing in aged subjects, who practice frequently sport activities, both for leisure and for counteracting with exercise metabolic diseases (i.e., diabetes and obesity) (Dallaudière et al., 2013). In this framework, it must be considered that aging itself may affect biochemical and biomechanical properties of tendons, so favoring the onset of tendon damage.

Non-insertional Achilles tendinopathy is a very common disease, mainly in sport-active population. Aim of this retrospective study was to compare the efficacy of PRP therapy in young and elderly subjects, addressed to our unit for recalcitrant non-insertional Achilles tendinopathy.

MATERIALS AND METHODS

Patients suffering from recalcitrant non-insertional Achilles tendinopathy treated beforehand in our Unit with PRP were retrospectively evaluated. Subjects who failed to respond to conservative treatments [e.g., eccentric training, laser, Extra-corporeal Shock Wawe, ultrasound (US), and steroid], with history of exercise-associated pain, pain or tenderness on palpation more than 3 months, and US features of chronic non-insertional damage in the Achilles' tendon were included. Exclusion criteria were: insertional Achilles tendinopathy, symptom duration <3 months, platelet values <150.000/mm3, Hemoglobin values < 11 g/dl, bleeding disorders, current use of anticoagulants or antiaggregants, hematological and rheumatic pathologies, severe systemic diseases (renal, hepatic, cardiac, infections, endocrinopathies, malignancies), immunodepression, and pregnancy (**Table 1**).

TABLE 1 | Inclusion and exclusion criteria.

Inclusion criteria	Exclusion criteria
Non-insertional Achilles tendinopathy	Insertional Achilles tendinopathy
Symptom duration >3 months	Symptom duration <3 months
No response to conservative treatments	Platelet values < 150.000/mm3
	Haemoglobin values < 11 g/dl
	Bleeding disorders
Ultrasound features of tendon damage	Use of anticoagulants/antiaggregants
	Rheumatic pathologies
	Severe systemic diseases
	Immunodepression
	Pregnancy

At baseline, in all subjects, demographic (age and sex) and anthropometric measures (height, weight, and BMI) were registered. Clinical (symptoms duration, weekly non-steroidal anti-inflammatory drugs consumption, associated diseases, sport practice) and functional data [Victorian Institute of Sports Assessment- Achilles questionnaire (VISA-A), adapted to the Italian language] were also collected. The VISA-A provides a subjective functional evaluation of Achilles tendon, and consists in eight questions which measure the domains of pain and function in daily living and sporting activity (Abate et al., 2013). Results range from 0 to 100, where 100 represent the perfect score.

Ultrasound evaluation was performed by the same well-trained operator (AM) using a high-resolution, multi-frequency (6–15 MHz) linear array transducer (*ProSound Alpha 10, Aloka, Japan*). Longitudinal and transverse scans were performed according to standard protocols (Maffulli et al., 2008) with the patient lying prone, with the feet hanging over the edge of the table at 90° of flexion. Loss of the normal fibrillar pattern, and/or irregularity of the tendon margins, and focal hypo-hyperechoic areas in the musculo-tendinous junction and/or in the mid-portion were considered as degenerative abnormalities. On the basis of these features tendons were stratified for severity as "mild" (one area of disorganized echotexture), "moderate" (some areas of disorganized echotexture), and "severe" (disorganized echotexture and diffuse hypo- or hyperechoic areas and/or calcifications). The presence of neovascularization, estimated by means of Color Doppler, was graded as (0), (1+), (2+), (3+), (4+), according to the appearance of vessels inside the tendon (Abate et al., 2013). To avoid artifacts, sensitivity was optimized for low flow, and color gain was set just below the noise level.

Platelet Rich Plasma was prepared using the Regen Lab A-PRP Kit. In detail, 8 ml of autologous blood was harvested from the cubital vein and collected into a tube containing a citrate anti-coagulant in addition to the thixotropic cell-separation gel. Then, the tube was carefully turn upside down several times (×5) to homogenize the blood with the anti-coagulant. After centrifugation [single spin, Force (RCF): 1500 *g*, 3400 rpm for 5 min], the blood was fractionated, with the red blood cells trapped under the gel, and the cellular sediment, including the platelets, settled on the surface of the gel. Therefore, by gently inverting the tube several time, the sediment was resuspended in the plasma supernatant, and PRP (4–5 ml, 1,6x native platelet concentration, >80% platelet recovery, no leukocytes, red blood cell remnant <0.3%) was obtained. PRP

TABLE 2 | Patients characteristics at baseline.

	Young	Elderly	*p*
Number	29	15	–
Tendons treated	36*	18**	–
Male:Female	19:10	13:2	–
Mean age	39.5 ± 6.9	61.5 ± 5.3	0.000
BMI	24.3 ± 1.8	25.9 ± 1.9	0.07
Symptoms duration (weeks)	28 ± 8.4	32.4 ± 6.5	0.1
Sport activities	20/29 (68.9%)	7/15 (46.6%)	0.14
Diabetes	–	3/15 (%)^	0.01
NSAIDs consumption	5/29 (17.2%)	3/15 (20%)	0.8
VISA-A	50.3 ± 8.8	48.7 ± 7.6	0.5

**7 and **3 bilateral. ^Well-controlled (self-report).*

was then collected into a 10 ml luer-lock syringe and ready for use.

After sterile dressing and under US control, small autologous PRP depots were left at several sites into the degenerate tendon areas, using a 21 Gage needle. PRP was placed at the site of most damaged areas, for a total amount of 4–5 ml. No regional anesthetic was used. A total of three injections (once a week) was performed. After the second injection, a rehabilitation program, based on eccentric training and stretching, was recommended daily (3 sets × 15 repetitions) at least for 3 months, during which a gradual return to sport activities was encouraged. After each injection, the patients were kept under observation for approximately 30 min (monitoring early side effects) and then discharged from the Unit. At home, patients were asked to restrict the use of the leg for at least 24 h; rest, ice packs, and acetaminophen (non-steroidal anti-inflammatory drugs were forbidden) were allowed. Moreover, patients were asked to register possible adverse events (pain, swelling, heat, functional limitations) and acetaminophen consumption during the following days after the injection.

Functional and instrumental evaluations were repeated after 1, 3, 6, and 12 months, and patients satisfaction was registered by means of five-points Likert Scale (Not at all satisfied; Slightly satisfied; Somewhat satisfied; Very satisfied; Extremely satisfied).

Statistical Analysis

According to age, patients were divided in two different cohorts (Young: <55 years old; Elderly: >55 years old. This partition was arbitrary done because a division universally accepted is not present). Demographic, US and clinical data, before and after treatment, were therefore compared.

Data are reported as mean ± standard deviation for continuous variables, whereas categorical and dichotomous variables are reported as frequencies and percentage. The significance level was determined at $p < 0.05$. The two-sample Student's *t*-test was used to compare continuous variables, when the distribution of data was normal; the Wilcoxon's rank sum test was used otherwise. The χ^2 test was used to evaluate associations between categorical data. All analyses were done using SAS statistical software, release 8.1.

RESULTS

Forty-four subjects met inclusion criteria. Demographic and clinical data of enrolled patients are reported in **Table 2**, which shows that, apart of age, and the presence of three cases of diabetes (well-controlled, self-report) in the elderly group, no differences were observed for other collected parameters. Neither at the instrumental examination, significant difference in the US degeneration and in the neovascularization score was present (**Table 3**).

No complications related to the injections or severe adverse events were observed during the treatment and follow-up period. Four young patients (2 at 3 months and 6 months, respectively) and three elderly subjects (2 at 3 and 1 at 6 months, respectively) were lost at follow-up.

The variations of VISA-A score at different follow-up times in the remaining patients of both groups are shown in **Table 4**; an increase of VISA-A score is observed both in young and elderly subjects throughout the whole length of the study, although with different levels of significance (higher in the young).

The infra-groups comparison shows that values increase more steadily and consistently in young patients, compared to the aged counterpart (**Figure 1**). At 12 months, 18/25 (72%) young and 6/12 (50%) elderly patients were very/extremely satisfied from the treatment. The US evaluation at 12 months, compared to baseline, did not show any significant variation in both groups.

DISCUSSION

The results of this study show that PRP treatment provides satisfactory results in young subjects with Achilles recalcitrant non-insertional tendinopathy reducing pain and improving function. These findings are in agreement with previous literature data in patients suffering from Achilles, patellar, and elbow tendinopathies (Andia and Abate, 2012; Andia and Maffulli, 2013; Andia et al., 2014). It is current opinion that the therapeutic activity of PRP is mainly due to the release of several growth factors (GFs), which can act on different aspects of tendon repair, including angiogenesis, chemotaxis, and

TABLE 3 | Ultrasound (US) findings at baseline in young and elderly patients.

	Young	Elderly	*p*
US degeneration			
Mild	10 (27.7%)	5 (27.7%)	0.7
Moderate	19 (52.7%)	9 (50%)	0.9
Severe	7 (19.4%)	4 (11.1%)	0.9
Neovessels			
Absent	6 (36.6%)	5 (27.7%)	0.5
Present	30 (83.3%)	13 (72.2%)	0.5
(1+)	8 (26.6%)	7 (53.8%)	0.1
(2+)	17 (56.6%)	4 (30.7%)	0.2
(3+)	3 (10%)	1 (7.6%)	0.7
(4+)	2 (6.6%)	1 (7.6%)	0.5

TABLE 4 | Victorian Institute of Sports Assessment- Achilles questionnaire (VISA-A) score during follow-up in young and elderly patients (intra-group comparison).

	VISA-A			
	Young	***p***	**Elderly**	***p***
Baseline	50.3 ± 8.8		48.7 ± 7.6	
1 month	56.8 ± 9.7	0.01	54.4 ± 7.1	0.04
3 months	68.7 ± 8	0.000	56.6 ± 4.9	0.002
6 months	72.4 ± 7.9	0.000	59.3 ± 8	0.000
12 months	76.1 ± 6.6	0.000	61.1 ± 9.4	0.000

cell proliferation by activating intracellular signal-transduction pathways (Anitua et al., 2006; de Mos et al., 2008; Abate et al., 2015).

In the short term (1–3 months), GFs can directly stimulate tenocytes to produce extracellular matrix, and promote neofibrils formation and remodeling. Indeed, it is well known that the Growth Hormone/Insulin-like GF-1 axis plays a central role in the regulation of human collagen turnover in musculo-tendinous tissue (Doessing et al., 2010; Andia and Abate, 2013). Insulin-like GF-1 stimulates collagen formation (Abrahamsson et al., 1991) and may also inhibit protein degradation, which is an effect of potential importance during immobilization periods when there is a net protein loss (Ye et al., 2013). PRP-released GFs and cytokines can also bind to fibrin and to proteoglycans in the extracellular matrix, constituting a storage pool that can be secondarily released by metalloproteases (Magra and Maffulli, 2005; Nurden, 2011). Actually, the mechanism of action is yet more complex, because the tissue outcomes may depend on the balance between plasma and platelet proteins (de Mos et al., 2008). However, the life-span of GFs and cytokines is relatively short even if repeated PRP injections can favor a better and long lasting action of platelet-derived GFs. So, it is conceivable that their effects can progressively fade until complete exhaustion.

Therefore, the persistent efficacy in the long-term (6–12 months), more than on a direct stimulation, probably relies on the activation of resident tendon stem/progenitor cells (TSPCs), which have been recently identified in tendons tissue from different animal species (Bi et al., 2007; Tempfer et al., 2009; Rui et al., 2010; Zhang et al., 2011; Mienaltowski et al., 2013). Like stem cells present in adult tissues, TSPCs are believed to be the source of newly differentiated tenocytes, responsible for maintaining adequate tenocyte numbers in the tissue throughout life and replenishing them after injury. Compared to bone marrow-derived mesenchymal stem cells, TSPCs express high levels of Scleraxis (a tendon-enriched specific transcription factor) and tenomodulin (a marker of adult tenocytes) and are able to form tendon and enthesis-like tissues when implanted *in vivo*. Morphologically, TSPCs possess smaller cell bodies and larger nuclei than ordinary tenocytes and have a cobblestone-like morphology in confluent cell cultures, whereas tenocytes are highly elongated, a typical phenotype of fibroblast-like cells (Zhang et al., 2011). TSPCs also proliferate more quickly than tenocytes in culture, and when implanted *in vivo* exhibit the ability to regenerate tendon-like tissues (Bi et al., 2007).

The biochemical niche, where TSPCs are embedded, is of paramount relevance for their appropriate maintenance and function. The importance of tendon extracellular matrix in the maintenance of TSPC stemness is supported by a recent study showing that rabbit Tendon Derived Stem Cells cultured on decellularized tendon matrix proliferated at a higher rate and had better stemness properties than those cultured on plastic tissue culture surface (Zhang et al., 2011). Moreover, GFs, as well as physiological loading, may increase TSPC numbers, by "awakening" or reactivating these cells (Sun et al., 2015). In conclusion, in young patients, it is

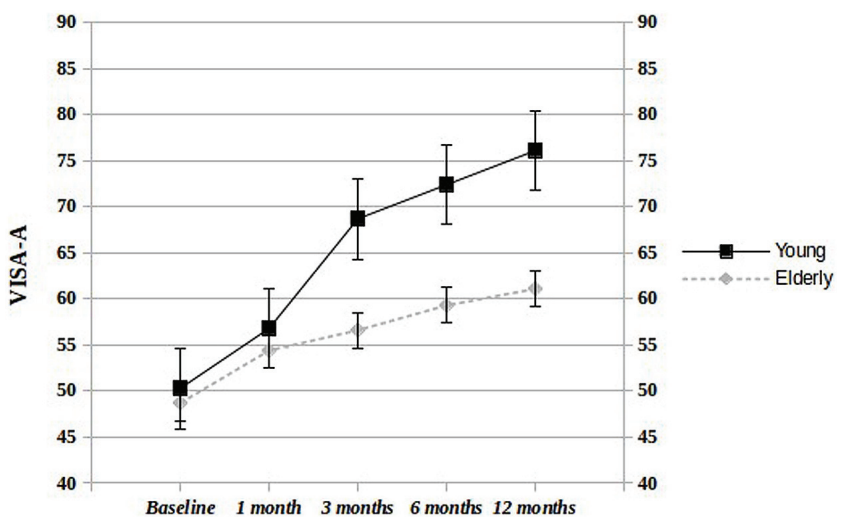

FIGURE 1 | Infra-group comparison at different follow-up times. Significant differences in VISA-A score were observed at 3, 6, and 12 months ($p < 0.000$) but not at 1 month ($p < 0.3$).

likely that PRP administration, associated to eccentric training exercises, may activate the resident stem-cells, assuring the prosecution of the healing mechanism for several months afterward.

In contrast with findings in young subjects, this study shows that the PRP injections promote positive response in the clinical parameters, although less evident, in aged people. In the present research, the elderly subjects at baseline had similar VISA-A scores, symptoms duration and US degeneration. However, whereas in the short term (after 1–3 months), they showed an increase of VISA-A scores, although less relevant than in young subjects, in the medium and long-term (6–12 months) no further significant clinical improvement was observed. These results are not surprising, taking into account different factors.

First, aging is associated with a decline of plasma levels of Insulin-like GF-1 (Rudman et al., 1981; Zadik et al., 1985; Leifke et al., 2000; Moller et al., 2009; Boesen et al., 2014), and platelets of aged people release minor amounts of GFs. In this respect, Cho et al. (2011) and Lohmann et al. (2012) found that mesenchymal stem cells' proliferation was higher with PRP from young donors, and that mesenchymal stem cells cultured with PRP from elder donors presented a senescent phenotype (Lohmann et al., 2012). Second, and most importantly, advanced age is associated to a numerical and/or functional deficits in resident populations of tenocytes and/or TSPCs. Senescent tenocytes become longer and thinner and have decreased protein synthesis, producing collagen fibers more disoriented with more variations in thickness. They also show a decrease in mucopolysaccharides, glycoaminoglycan, chondroitin sulfate, dermatan sulfate and in water content (Ippolito et al., 1980; Riley et al., 1994; Arnesen and Lawson, 2006; Thorpe et al., 2010; Ruzzini et al., 2014). Moreover, senescent cells are characterized by the elevated expression of senescent cell markers (β-gal), senescence-associated genes (p53, p21, and p16INK4a, metalloproteases, ADAMTS), and pro-inflammatory cytokines (Dimri et al., 1995; Campisi and d'Adda di Fagnana, 2007; Russo et al., 2015). Age-related changes in tenocyte behavior can be also responsible for altered migration and proliferation rate. Tsai et al. (2011) in an *in vitro* experiment performed on tenocytes derived from young, middle-age and old Sprague–Dawley rats, showed that decline in proliferation is directly correlated to aging and that aged tenocytes tend to stop in G0/G1 cellular phase. These results have been confirmed by several authors (Thorpe et al., 2010; Klatte-Schultz et al., 2012; Kostrominova and Brooks, 2013; Torricelli et al., 2013). Aged TSPC show similar characteristics, namely a profound TSPC self-renewal deficit accompanied with premature entry into cellular senescence; significant changes in the expression of genes regulating cell adhesion, migration, cytoskeleton (scleraxis and tenomodulin), dysregulated cell-matrix interactions and actin dynamics have been also observed (Kohler et al., 2013; McCharty and Hannafin, 2014). Interestingly, Zhou et al. (2010) showed that aged TSPCs formed adipocytes more readily than younger cells and expressed higher levels of adipogenic markers (PPARγ2, C/EBPa, and leptin) following induction. These data may help to explain the higher levels of adipose tissue normally associated with older tendons (Kannus and Jozsa, 1991), a pattern similar to that observed in bone marrow, where adiposity was found to correlate inversely with the functionality of hematopoietic stem/progenitor cells (Naveiras et al., 2009).

Therefore, a large number of concordant and sound biological data may explain why in aged persons PRP preparations can be less effective in promoting the activation of tenocytes and their progenitors cells, and therefore long-term tissue healing.

The findings of the present study can be hardly compared to those found in literature. Indeed, only few authors evaluated the impact of age on the therapeutic response to PRP (Ferrero et al., 2012; Boesen et al., 2014; Filardo et al., 2014). In these studies age resulted not influent on the outcomes, but the mean age of the patients was significantly lower, and aged persons were under-represented in the samples.

Some limitations of our research must be acknowledged. First, as well as for all the studies on PRP therapeutic activity, a key aspect to consider is the composition of the product used, because it cannot be excluded that other PRP formulations, different for cell type content, platelet concentration, storage modalities, activation methods, and protocol for therapeutic applications, could be more beneficial in aged people (Abate et al., 2012; Andia and Abate, 2012). Second, the data were retrospectively collected. However, in this respect, it must be observed that the patient's selection was very careful and only those who had specific inclusion/exclusion criteria and were followed with a fixed experimental protocol were evaluated. Third, activity scales (i.e., Tegner Scale) was not collected; at this regard, it must be noted that sedentary lifestyle, which is more common in elderly subjects, may negatively influence the main outcomes of the study. In the future, a prospective trial, using PRP with higher platelet concentration, or with the addition of exogenous GFs, and enrolling a consistent number of subjects, will allow more sound and definitive conclusions.

Ethical Statement

All procedures performed in study involving human participants were in accordance with the ethical standards of the institutional and/or national committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all participants included in the study.

AUTHOR CONTRIBUTIONS

VS: Design of the work; critical revision of the paper; final approval of the version to be published; agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

DV: Paper revision; final approval of the version; agreement to be accountable for all aspects of the work in ensuring that

questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

AP: Paper revision; final approval of the version; agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

MA: Design of the work, acquisition, analysis, and interpretation of data; drafting and critical revision of the work; final approval of the version to be published; agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Tendon Extracellular Matrix Alterations in Ullrich Congenital Muscular Dystrophy

*Francesca Sardone[1,2], Francesco Traina[3], Alice Bondi[3], Luciano Merlini[4], Spartaco Santi[2,5], Nadir Mario Maraldi[2,5], Cesare Faldini[3] and Patrizia Sabatelli[2,5]**

[1] Department of Biomedical Sciences, University of Padova, Padova, Italy, [2] National Research Council of Italy, Institute of Molecular Genetics, Bologna, Italy, [3] Rizzoli Orthopaedic Institute, University of Bologna, Bologna, Italy, [4] Muscle Clinic, Villa Erbosa Hospital, Gruppo San Donato, Bologna, Italy, [5] SC Laboratory of Musculoskeletal Cell Biology, IOR-IRCCS, Bologna, Italy

Collagen VI (COLVI) is a non-fibrillar collagen expressed in skeletal muscle and most connective tissues. Mutations in COLVI genes cause two major clinical forms, Bethlem myopathy and Ullrich congenital muscular dystrophy (UCMD). In addition to congenital muscle weakness, patients affected by COLVI myopathies show axial and proximal joint contractures and distal joint hypermobility, which suggest the involvement of the tendon function. We examined a peroneal tendon biopsy and tenocyte culture of a 15-year-old patient affected by UCMD with compound heterozygous *COL6A2* mutations. In patient's tendon biopsy, we found striking morphological alterations of tendon fibrils, consisting in irregular profiles and reduced mean diameter. The organization of the pericellular matrix of tenocytes, the primary site of collagen fibril assembly, was severely affected, as determined by immunoelectron microscopy, which showed an abnormal accumulation of COLVI and altered distribution of collagen I (COLI) and fibronectin (FBN). In patient's tenocyte culture, COLVI web formation and cell surface association were severely impaired; large aggregates of COLVI, which matched with COLI labeling, were frequently detected in the extracellular matrix. In addition, metalloproteinase MMP-2, an extracellular matrix-regulating enzyme, was increased in the conditioned medium of patient's tenocytes, as determined by gelatin zymography and western blot. Altogether, these data indicate that COLVI deficiency may influence the organization of UCMD tendon matrix, resulting in dysfunctional fibrillogenesis. The alterations of tendon matrix may contribute to the complex pathogenesis of COLVI related myopathies.

Keywords: Collagen VI, Ullrich congenital muscular dystrophy (UCMD), pericellular matrix, metalloproteinases, tendon

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****Correspondence:***
Patrizia Sabatelli
sabatelli@area.bo.cnr.it

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INTRODUCTION

Tendons are composed of relatively rare cells (tendon fibroblasts) scattered within a predominant dense connective tissue arranged in an highly ordered ECM, mainly constituted by collagen fibrils, which are hierarchically organized to withstand tensile forces transmitted from muscles to bone axis (Kadler et al., 1996). Fibrils contain mostly collagen I (COLI) and other components, which contribute to fibrillogenesis, such as collagen types III, V, VI, XII, and XIV, as well as proteoglycans and glycoproteins (Screen et al., 2015).

Fibril assembly is crucial for tendon function. Fibril intermediates assemble by lateral and longitudinal association (Birk et al., 1995). The assembly of intermediates into longer, continuous fibrils with larger diameters dramatically increases the tensile strength of tissues such as the tendon. Tendon fibrillogenesis is regulated by a variety of fibril surface associated molecules, as fibronectin (FBN), decorin, biglycan and collagens types XII and XIV (Birk et al., 1995; Young et al., 2002; Zhang et al., 2005). These regulatory matrix molecules are all substrates for MMP-2/MT3-MMP, which ensures the matrix turnover required during tendon development (Jung et al., 2009).

Collagen VI (COLVI) is a microfibrillar collagen expressed in most tissues. In tendons and ligaments, COLVI forms a network of beaded filaments associated both to collagen fibrils and to the cell surface (Bruns et al., 1986; Ritty et al., 2003). The best-characterized and widely expressed form of COLVI is the [α1, α2, α3] heterotrimer that further assembles intracellularly into dimers and tetramers. After secretion, tetramers undergo end-to-end association, giving rise to the typical 100 nm-spaced beaded microfibrils (Bruns et al., 1986), which may form, alternatively, fibrils by parallel alignment, or web-like structures by multiple interconnections, depending on the association with cell receptors and ECM-binding proteins (von der Mark et al., 1984; Bruns et al., 1986; Wiberg et al., 2002; Knupp et al., 2006; Koudouna et al., 2014). In humans, two novel COLVI subunits, the α5 and α6 chains, were recently identified, which structurally resemble the α3 chain but display a more restricted and often alternative distribution pattern (Fitzgerald et al., 2008; Gara et al., 2008; Sabatelli et al., 2011, 2012). In tendons, the [α1, α2, α3] heterotrimer is abundantly expressed (Thakkar et al., 2014), whereas the α5 chain is selectively detected at the myotendinous junction and the α6 chain is absent (Sabatelli et al., 2012).

Mutations in the genes encoding COLVI (*COL6A1, COL6A2, and COL6A3*) cause the COLVI-related myopathies, which comprise two major clinical forms, Bethlem myopathy (BM [MIM 158810]) and Ullrich congenital muscular dystrophy (UCMD [MIM 254090]), and the limb girdle and the Myosclerosis myopathy (MM) variants. UCMD is a severe disorder characterized by congenital muscle weakness; BM is a mild form characterized by slowly progressive axial and proximal muscle weakness; MM is characterized by slender muscles with firm "woody" consistence and restriction of movement of many joints (Merlini and Bernardi, 2008). COLVI myopathies are also characterized by joint hyperlaxity and contractures. Type and distribution of contractures are distinguishing features of COLVI disorders. The UCMD is characterized by proximal contractures and distal laxity, BM by distal contractures, limb-girdle phenotype by late or no contractures, and MM by early, diffuse, and progressive muscle contractures resulting in severe limitation of movement of all axial, proximal, and distal joints (Merlini and Bernardi, 2008). Patients affected by COLVI myopathies may also display skin abnormalities, like keloids or "cigarette article" scars, dry skin, striae rubrae, and keratosis pilaris (follicular keratosis). Thus, COLVI mutations result in disorders with combined muscle and connective tissue involvement.

Animal models of COLVI myopathies have been developed in mice (Bonaldo et al., 1998; Pan et al., 2013) and zebrafish (Telfer et al., 2010; Zulian et al., 2014). Recently, a nonsense variant in *COL6A1−/−* has been detected in Landseer dogs (Steffen et al., 2015). *COL6A1* mice, a COLVI null model (Bonaldo et al., 1998), and *COL6A3* deficient mice (Pan et al., 2013) develop a mild myopathy and tendon dysfunction possibly due to altered tendon fibrillogenesis (Izu et al., 2011; Pan et al., 2013). *COL6A1* morphant zebrafish display a severe impairment of motor function and myotendinous junction abnormalities (Telfer et al., 2010; Zulian et al., 2014). Altogether, these data point to an involvement of COLVI in the regulation of tendon function.

In order to determine whether the altered tendon fibrillogenesis reported in animal models of COLVI myopathies is also present in humans, we studied a tendon biopsy and tenocyte cultures of a UCMD patient with compound heterozygous mutations in *COL6A2* gene (Martoni et al., 2009). We found changes consistent with altered fibrillogenesis, as indicated by fibrils abnormalities, and *in vitro* alterations of COLI organization and metalloproteinase MMP-2 activity.

MATERIALS AND METHODS

Tendon Biopsies

Peroneal tendon biopsies were harvested from two healthy subjects (17 and 21) during foot surgery and during a Grice procedure from a previously genetically characterized UCMD patient carrying compound heterozygous mutation for a G > A variation at position +5 of *COL6A2* intron 8 and a nonsense mutation R366X in *COL6A2* exon 12 (Martoni et al., 2009). All patients granted informed consent. Tendon fragments were subjected to mechanical dissociation, and maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 1% antibiotics plus 10% Fetal Bovine Serum (FBS; Nemoto et al., 2013); 0.25 mM L-ascorbic acid was added to the medium to allow COLVI tetramer secretion (Engvall et al., 1986).

Immunofluorescence and Confocal Analysis

The immunofluorescence analysis with anti-COLVI (Millipore) on tendon cell cultures was performed as previously reported (Sabatelli et al., 2012). Cells grown onto coverslips were incubated with antibodies against FBN (Sigma), COLVI (Millipore), COLI (Abcam), and with FITC or TRITC-conjugated anti-mouse or anti-rabbit secondary antibodies (DAKO). Cell nuclei were stained with 1 mg/ml DAPI (Sigma-Aldrich). Samples were mounted with an anti-fading reagent (Molecular Probes). The confocal imaging was performed with a Nikon A1-R confocal laser scanning microscope, equipped with a 60×, 1.4 NA objective and with 405, 488 and 561 nm laser lines to excite DAPI (blu), FITC and TRITC fluorescence signals. Each final confocal image, of 1024 × 1024 pixels and 4096 gray levels, was obtained by maximum intensity projection of 10 optical

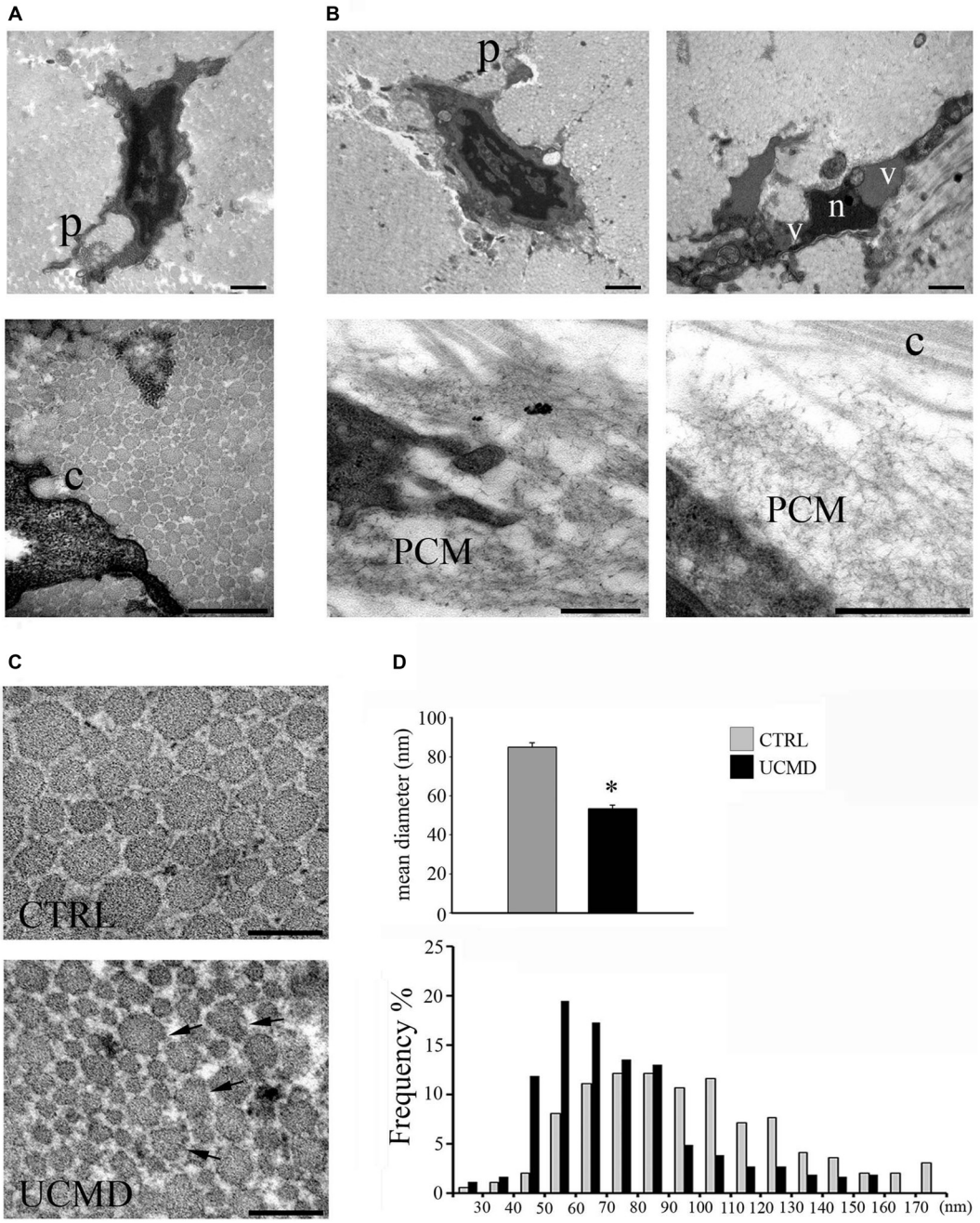

FIGURE 1 | (A) Ultrastructural analysis of normal peroneal tendon showing a tenocyte with cellular processes (p, upper panel) which define extracellular matrix compartments containing collagen fibrils. Collagen fibrils are closely associated with the cell membrane (c, lower panel). Scale bar, 1 μm. **(B)** Ullrich congenital muscular dystrophy (UCMD) tenocytes display reduced and irregular cellular processes (p). A necrotic cell with hypercondensed heterochromatin (n) and vacuoles (v) is shown (upper lane, right panel). Note the presence of abnormal microfibrillar material accumulated in the pericellular matrix (PCM) of UCMD fibroblasts. Scale bar, 1 μm. **(C)** Transmission electron microscopy of cross-sectioned normal and UCMD peroneal tendon. Normal tendon displays fibrils with regular profile. In contrast, UCMD tendon displays smaller diameter fibrils; several aberrant fibrils with irregular profile are also observed (arrows). Scale bar, 200 nm. **(D)** Fibril diameter distribution in normal and UCMD tendon. The fibrils diameter distribution was shifted toward smaller diameters in UCMD tendon compared to normal tendon, with a significant difference in mean fibril diameter (± SEM $^*p < 0.001$).

sections passed through the central region of the cells (recorded at *z*-step size of 300 nm). Volume view with 3D rendering was carried out using the NIS Elements Advanced Research Software (Nikon).

Western Blot Analysis

Cultured tendon fibroblasts were harvested by scraping. The media were recovered after cell treatment with 0.25 mM L-ascorbic acid for 24 h without FBS, and concentrated with

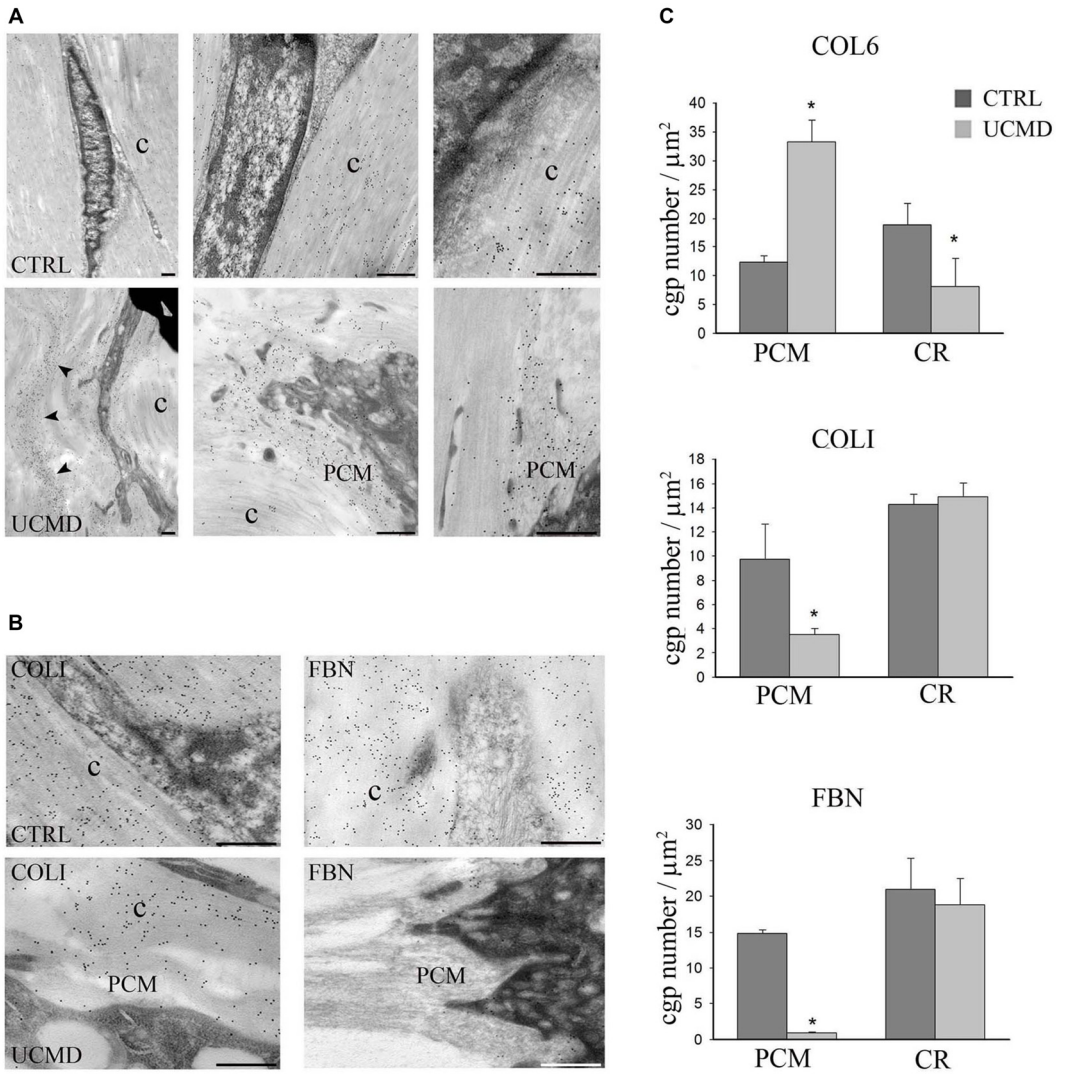

FIGURE 2 | (A) Immunoelectron microscopy analysis of collagen VI (COLVI) on tissue sections of normal (upper row) and UCMD (lower row) peroneal tendon sections. In normal tendon, COLVI, identified by 15 nm colloidal gold particles, is detected among collagen fibrils (c). In contrast, in UCMD tendon, COLVI appears concentrated in the pericellular matrix of tenocytes (PCM) and scarcely associated with the collagen fibrils (c). Scale bar, 200 µm. **(B)** Immunoelectron microscopy of collagen I (COLI) (left panels) and fibronectin (FBN; right panels) in normal (upper panels) and UCMD (lower panels), showing a marked reduction of COLI and FBN in the pericellular matrix (PCM) of UCMD tenocytes compared to normal control (c, collagen fibrils). Scale bar, 200 nm. **(C)** Quantitative analysis of the density of colloidal gold particles (cgp) in the pericellular matrix (PCM), and in ECM displaced from the cells (central region, CR) of the UCMD and normal control tendon labeled with anti-COLVI, COLI and FBN antibodies. (± SEM $^*p < 0.001$).

Vivaspin sample concentrators (Vivaspin 2 MWCO10000, GE Healthcare) according to the manufacturer's operating procedures. Cell lysates and concentrated culture media were resolved by standard SDS–PAGE, electroblotted onto a nitrocellulose membrane (Sardone et al., 2014) and incubated with antibodies against FBN (Sigma), COLI (Abcam), tenomodulin (TNMD; Santa Cruz), actin (Santa Cruz), MMP2 (Santa Cruz), followed by incubation with anti-mouse or anti-rabbit horseradish peroxidize (HRP)-conjugated secondary antibodies. Chemiluminescent detection of proteins was carried out with the ECL detection reagent Kit (GE Healthcare Amersham, Pittsburgh, PA, USA) according to the supplier's instructions.

Gelatin Zymography

The activity of MMP-2 and MMP-9 in conditioned medium was detected using gelatin zymography, which was performed under non-reducing conditions in a 7.5% SDS-polyacrylamide gel containing 2 mg/ml gelatin (Mini-PROTEAN II system; Bio-Rad Laboratories Ltd, Hempstead, UK). Gels were washed in 2.5% Triton X-100 to remove SDS and allow renaturation of MMPs, before they were transferred to a solution containing 50 mM Tris (pH 7.5), 5 mM $CaCl_2$, and 1 mM $ZnCl_2$, followed by incubation at 37°C for 18 h. After staining with Coomassie brilliant blue R250 (Bio-Rad Laboratories, Hercules, CA, USA), pro-MMPs and active MMPs were observed as white lysis bands produced by gelatin degradation.

Electron Microscopy Study

Tendon fragments were fixed with 2.5% glutaraldehyde in 0.1 M cacodilate buffer, postfixed with 1% osmium tetroxide in 0.1 M cacodilate buffer and embedded in Epon812 epoxy resin following standard procedures. For post embedding immunoelectron microscopy, tendon fragments were fixed with 1% glutaraldehyde in phosphate buffer, embedded in London white resin and ultrathin sections were incubated with an anti-COLVI (Fitzgerald et al., 2008), anti-COLI (Abcam) and anti-FBN (Sigma) as previously reported (Sabatelli et al., 2011), and revealed with anti-rabbit 15 nm colloidal gold conjugated antibody (Sigma). Sections were stained with uranyl acetate and lead citrate and observed with a Jeol JEM-1011 transmission electron microscope operated at 100 kV. For quantitative analysis of COLVI, COLI and FBN immunogold labeling, at least 20 fields for each sample were acquired at the same magnification, and the labeling density was expressed as mean of the number of gold particles/$\mu m^2 \pm$ SD.

Statistical Analysis

Statistical analysis was performed by Student's *t*-test with the Statistical Package for the Social Sciences Software (SPSS, Chicago, IL, USA). The results were considered statistically significant for p values less than 0.05.

RESULTS

By ultrastructural analysis, normal peroneal tendons showed the presence of scattered tenocytes with long cellular processes and well-packed and oriented collagen fibrils. The tendon matrix appeared mainly constituted by collagen fibrils of different size and few scattered elastin-oxytalan fibers (**Figure 1A**). Collagen fibrils were present in the pericellular matrix of tenocytes, closely associated with the cell surface (**Figure 1A**, lower panel).

In contrast, patient's tenocytes showed reduced cell processes, and occasionally, displayed features of necrotic cells, such as hypercondensed heterochromatin and increased vacuoles (**Figure 1B**). Numerous tenocytes displayed an abnormal accumulation of microfibrillar/reticular material in the pericellular matrix, which determined the displacement of collagen fibrils from the cell surface (**Figure 1B**). In addition, cross-sectioned collagen fibrils displayed irregular profiles and a ragged appearance (**Figure 1C**). The analysis of the fibril diameter distribution revealed a reduced size of patient's fibrils compared with normal tendon, with an increased number of small fibrils (20–60 nm) and reduced number of large fibrils (>100 nm; **Figure 1D**); moreover, the mean fibril diameter was significantly reduced (**Figure 1D**).

In addition, immunoelectron microscopy study showed that the colloidal gold particles, identifying COLVI, had an uneven distribution in the patient's matrix tendon, with a conspicuous accumulation in the pericellular matrix of tenocytes and in focal areas of the tendon matrix (**Figure 2A**); moreover, the association of COLVI with collagen fibrils bundles

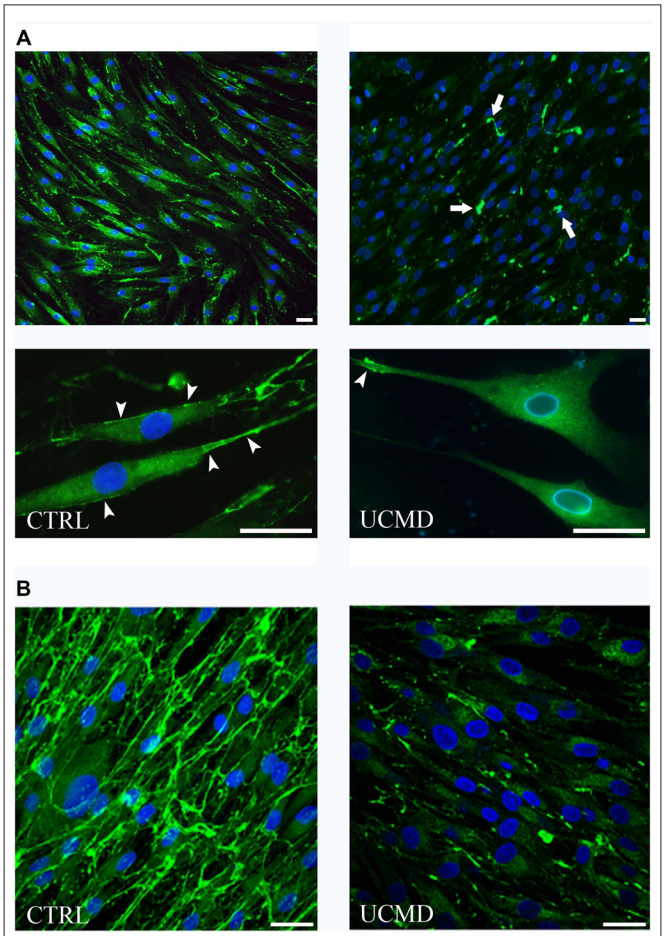

FIGURE 3 | (A) Immunofluorescence microscopy of COLVI antibody in normal (CTRL, left panels) and UCMD patient (UCMD, right panels) tenocyte cultures treated with ascorbic acid for 24 h. COLVI is early secreted in normal tenocyte culture and associates with the cell surface (arrowheads). In UCMD tenocyte culture, COLVI is severely reduced in the matrix and form anomalous aggregates (arrows). The association of COLVI with the cell surface is also impaired. Nuclei were stained with DAPI (blue). Scale bar, 20 μm. **(B)** Immunofluorescence microscopy of COLVI in normal (CTRL, left panel) and UCMD (right panel) long term tenocyte cultures showing the abnormalities of COLVI organization in patient sample compared to complex network developed in normal culture. Nuclei were stained with DAPI (blue). Scale bar, 20 μm.

was apparently impaired compared with the intense labeling detected in normal tendon matrix. In addition, immunoelectron microscopy study of COLI and FBN, both COLVI-related components of tendon matrix, showed a marked reduction in the pericellular matrix of UCMD tendon fibroblasts (**Figure 2B**). These observations were supported by the quantitative analysis of the number of colloidal gold particles both in the PCM and in areas of tendon matrix displaced from the cells, which further demonstrated significant changes of COLVI, COLI and FBN distribution in the PCM of the UCMD tendon biopsy (**Figure 2C**).

To address further the impact of COLVI deficiency on tendon matrix organization, we studied normal and UCMD patient tendon cultures by immunofluorescence microscopy.

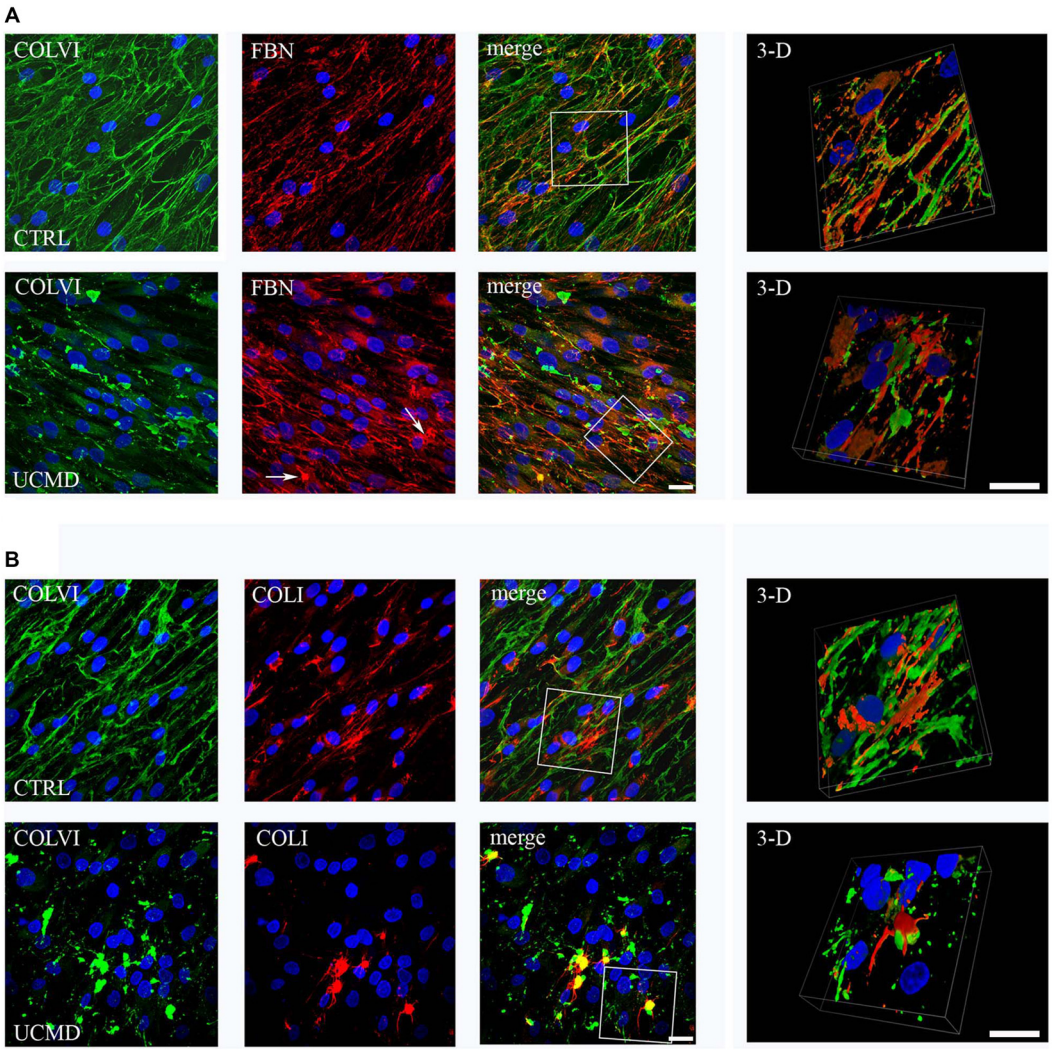

FIGURE 4 | (A) Confocal microscopy of (COLVI) and FBN in normal (upper row) and UCMD patient (lower row) tenocyte cultures; 3D surface shaded reconstruction of an enlargement of the area defined by the white box is shown on the right. In normal tendon fibroblast culture, COLVI network co-localizes at discrete site with FBN, as visualized in merge and 3D reconstruction images. In UCMD sample, COLVI forms anomalous aggregates, which also include FBN staining (arrows). Nuclei were stained with DAPI (blue). Scale bar, 50 μm. **(B)** Confocal microscopy of (COLVI) and COLI in normal (upper row) and UCMD patient (lower row) tendon fibroblast cultures; 3D surface shaded reconstruction of an enlargement of the area defined by the white box are shown on the right. In normal tendon fibroblast culture, COLI and COLVI form distinct interconnected networks, as indicated by the partial association visualized in 3D reconstruction. In UCMD tendon culture, COLI forms aggregates that match with COLVI abnormal structures. 3D reconstruction of a particular of the merge image clearly shows that collage I associates with COLVI aggregates. Nuclei were stained with DAPI (blue). Scale bar, 50 μm.

Normal and UCMD tenocyte cultures were grown to confluence onto coverslips and treated for different times with ascorbic acid to assess early (24 h) and late (10 days) dynamics of COLVI extracellular assembly. Immunofluorescence microscopy of normal tenocyte cultures showed that COLVI was mainly associated with the cell surface in short term cultures (**Figure 3A**), while, in long-term samples, it formed a complex and well-developed network (**Figure 3B**). In UCMD tenocyte culture, COLVI was markedly reduced and poorly associated to the cell surface (**Figure 3A**); when analyzed in long term cultures, COLVI displayed a spot-like pattern, with large aggregates scattered in the extracellular matrix (**Figure 3B**).

Double labeling of anti-COLVI with anti-FBN revealed moderate changes of the FBN pattern in UCMD tenocyte culture compared to normal control; in fact, small FBN aggregates, which co-localized with COLVI labeling, were detected (**Figure 4A**). In contrast, COLI organization was severely affected in UCMD culture, as indicated by anomalous aggregates which matched with COLVI deposits (**Figure 4B**).

Western blot analysis of cell lysates and conditioned medium showed comparable amount of FBN in UCMD and control tenocyte cultures, as indicated by densitometric quantification (**Figure 5A**); COLI was normally expressed in cell lysate, while

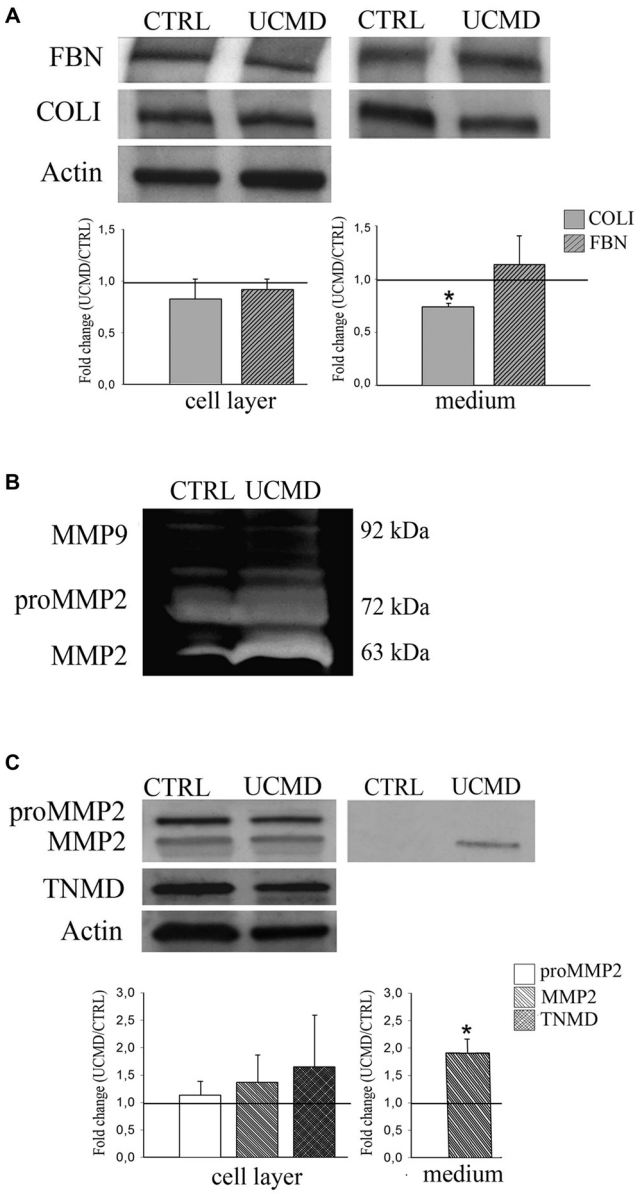

FIGURE 5 | (A) Western blot analysis of cell lysate (Cell Layer) and conditioned medium (Medium) of normal (CTRL) and UCMD patient (UCMD) cultured tenocytes of FBN and COLI, and of the relative densitometric quantification; actin was used as a loading control of cell lysates. For cell lysate quantification, protein levels were calculated as relative intensity with respect to actin. The respective protein levels in UCMD patient cells and media were compared to the control (showed by the dark line, set as 1) ± SD (*$p < 0.06$ vs. control). **(B)** Gelatin zymography of conditioned medium from normal (CTRL) and UCMD patient showing an increased gelatinolitic activity of MMP2 in the culture medium of patient cells. **(C)** Western blot analysis with anti-MMP2 antibody, recognizing both the active (63 kDa) and non-active pro-MMP2 (72 kDa) form in normal (CTRL) and UCMD patient (UCMD) cultured tenocytes (Cell Layer) and conditioned medium (Medium), and the relative densitometric quantification; actin was used as a loading control of cell lysates, while tenomodulin (TNMD), a tenocyte marker, was used to assess the cell phenotype. For cell lysates, protein levels were calculated as relative intensity with respect to actin. The respective protein levels in UCMD patient cells and media were compared to the control (showed by the dark line, set as 1). Error bars indicate SD. (*$p < 0.001$ vs. control).

it was moderately reduced in the patient conditioned medium (**Figure 5A**). Furthermore, we investigated the expression and activity of gelatinases MMP2 and MMP9, both involved in tendon matrix turnover (Veidal et al., 2011). Strikingly, gelatin zymography of conditioned medium of patient tendon fibroblasts displayed an increased activity of MMP2, while pro-MMP2 and MMP9 activity were similar to that detected in the control conditioned medium (**Figure 5B**). Consistent with increased MMP2 gelatinolytic activity, western blot analysis showed an increase of the 63 kDa active form of MMP2 in the conditioned medium of patient tenocytes, while pro-MMP2 was unchanged (**Figure 5C**). The analysis of MMP2 in cell lysates did not showed differences between UCMD and normal tendon cultures.

DISCUSSION

Patients with mutations in COLVI genes develop both contractures and distal laxity, possibly due to tendon/ligament involvement. However, the molecular basis and the mechanism leading to these alterations remain unknown. In this article, we report for the first time the impact of COLVI deficiency on the organization of tendon matrix of an UCMD patient, represented by morphological alterations of tendon fibrils, and disruption of tenocyte pericellular matrix organization associated with increased MMP2 activity.

COLVI was markedly reduced both in tendon sections and tenocyte culture of the UCMD patient. This alteration is consistent with the low protein level and the derangement of the COLVI network previously reported in skeletal muscle (Tagliavini et al., 2014) and skin fibroblast cultures of the same patient (Martoni et al., 2009). On the other hand, the majority of COLVI mutations of UCMD patients affect the assembly and secretion of COLVI (Zhang et al., 2002; Merlini and Bernardi, 2008). This may depend on the complex mechanism of COLVI assembly, a multistep process, which involves intracellular formation of dimers and tetramers before secretion (Bruns et al., 1986).

The COLVI quantitative defect was associated to striking alterations of fibrils organization, both in tissue sections and tenocyte cultures. The immunoelectron microscopy analysis of tendon sections showed that COLVI was abnormally accumulated in the pericellular matrix of the patient's tenocytes and in focal areas of the extracellular matrix, rather than associated to fibrils, as in normal tendon (Bruns et al., 1986). Similarly, aberrant COLVI aggregates were detected in patient's tenocyte culture, indicating that the low amount of protein secreted is not able to organize a regular matrix. It is interesting to note that in UCMD patients, COLVI may be reduced in the basal lamina surrounding the muscle fibers, but is still present/accumulated in the endomysium and perivascular space (Pan et al., 2003). Aggregates of COLVI have also been reported in the skin (Sabatelli et al., 2011) and in fibroblast cultures of UCMD patients (Hicks et al., 2008; Martoni et al., 2013). It has been proposed that mutant COLVI is not degraded but is aberrantly accumulated in the interstitial space. As possible consequence, COLVI non-functional deposits may hinder the

arrangement of extracellular binding partners. Consistent with this hypothesis, the expression of FBN and COLI was severely affected in areas of COLVI accumulation, and in particular in the pericellular matrix of tenocytes, as demonstrated by immunoelectron microscopy on patient's tendon.

The effect of aberrant COLVI expression on early assembly of COLI and FBN, was better characterized by studying two-dimensional tenocyte cultures. We found that COLI, and to a lesser extent, the FBN organization were affected in patient's tenocyte cultures. Remarkably, COLI failed to organize a filamentous network aligned to the cell axis, compared to the well-oriented fibrils assembled by normal tenocytes. These data may suggest that collage VI is involved in directional deposition of COLI. It is interesting to note that COLVI in normal cultured tenocytes is associated to the cell surface, and that this pattern was impaired in patient's culture, pointing to a role of COLVI in cell-surface associated mechanisms, as early steps of collagen fibril assembly (Zhang et al., 2005). The alteration of the FBN pattern in cultured tenocytes is consistent with our previous reports that COLVI deficiency affects the three-dimensional organization of FBN in fibroblast cultures of UCMD and Bethlem myopathy patients (Martoni et al., 2009), and in *Col6A1-/-* null mice (Sabatelli et al., 2001).

The ultrastructural analysis of UCMD tendon showed alterations of fibril morphology and a significant reduction of the number of large fibrils. These data correlated with fibril abnormalities reported in skin of UCMD patients (Kirschner et al., 2005), and in tendons of COLVI myopathy mouse models (Izu et al., 2011; Pan et al., 2013, 2014), and further support the hypothesis of dysfunctional fibrillogenesis.

COLVI interacts with a large number of regulatory molecules, including metalloproteinase MMP-2 (Freise et al., 2009). Interestingly, we found a specific increase of MMP-2 gelatinolytic activity, consistent with the increase of active MMP-2 in the medium of UCMD cultured tenocytes. It is interesting to note that COLVI, and in particular the α2 chain, modulates the activity of MMP-2 by sequestering pro-MMPs in the extracellular matrix, and blocking proteolytic activity (Freise et al., 2009). A moderate increase of MMP-2 activity has been observed in *Col6A1-/-* mice, a COLVI null model (Izu et al., 2011). MMP-2 is involved in the initiation and progression of fibril growth and matrix assembly during tendon development (Jung et al., 2009); increased level of MMP2 has been also reported during tendon healing (Choi et al., 2002). We hypothesize that MMP-2 increased activity may reflect an accelerated tendon matrix turnover in response to defects of COLVI.

Altogether, our data indicate that COLVI deficiency affects both *in vivo* and *in vitro* the organization of matrix tendon, resulting in dysfunctional fibrillogenesis. Fibril alterations have been reported in some forms of Ehlers Danlos Syndrome (EDS) with hypermobile phenotype (Kobayasi, 2004) and in an animal model of EDS with joint phenotype (Sun et al., 2015), suggesting common pathophysiological pathways in this group of connective tissue disorders.

Fibril abnormalities are also regarded as a consequence of decreased loading (Heinemeier and Kjaer, 2011), disuse, and aging sarcopenia (Narici and Maganaris, 2007). Our data, however, point toward a primary tendon dysfunction. In fact, we have shown that alterations of the extracellular matrix were also present in the patient's cultures, effectively reducing the importance of muscle dysfunction as a determinant of the tendon phenotype.

AUTHOR CONTRIBUTIONS

FS performed in culture studies; PS performed the immunogold and the ultrastuctural study; SS performed the confocal analysis; AB, FT, CF, LM and NMM participated in data collection, data interpretation, and reviewed and critiqued the manuscript. All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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